Author:
Fudulu, Daniel

Title:
Characterisation of the stress response to heart surgery in children

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Daniel Fudulu

"A dissertation submitted to the University of Bristol in accordance with the requirements for the award of the degree of Doctor of Philosophy in the Faculty of Health Sciences, September 2019."

Word count: 50115

1 ABSTRACT

In this PhD thesis, I investigate the importance of inflammatory cytokines for activation of adrenal activity, and couple this with the use of a novel microdialysis system to assess the response of the hypothalamic-pituitary-adrenal axis in children undergoing cardiac surgery. The thesis is structured in three parts.

Following the recent demonstration that there is an increased sensitivity of the adrenal cortex to ACTH in adults undergoing cardiac surgery, I investigated whether this could be mediated by cytokines. In the first part of my thesis, I describe a novel co-culture model to investigate the local, immune-adrenal crosstalk and its impact on steroidogenesis. I demonstrate that in the adrenocortical cell line ATC-7 the expression of pro-inflammatory cytokines after LPS stimulation is dependent on the ratio of adrenal and immune cells, that the presence of immune cells can modulate steroidogenesis and that this interaction can be further modulated by ACTH stimulation. In the second part, I discuss the current evidence and knowledge gaps behind the practice of glucocorticoid administration in paediatric heart surgery. I also report the results of a national survey showing a considerable variation in glucocorticoid administration within and between units.

The above findings set the scene for the third part of this dissertation, that reports a study protocol for the basic understanding of the HPA axis function in children undergoing paediatric heart surgery the - Peacock Study. By using our novel automated tissue microdialysis system, I successfully measured tissue cortisol and cortisone levels in children of various ages without reducing their circulating blood volume. I also report the preliminary results of the study on 36 children undergoing surgery or catheter procedures. Firstly, I demonstrate that cortisol secretion in children is secreted with a pulsatile pattern, suggesting single-point cortisol testing is obsolete. Furthermore, I found marked changes in the perioperative cortisol to cortisone ratio. Neonates had a distinct response in that they had lower cortisol to cortisone rations presumably related to differential 11 β-HSD isoenzymes activities. Also, I show that the release of CBG and cytokines is dependent on the age of the child, the type of procedure and preoperative oxygen saturation.

2 DEDICATION

I dedicate this thesis to my father, Professor Paul Fudulu. He is the first solid and uncompromising researcher I have met early on in my life. Our discussions about his research that tries to link economics (regarded as a social science) with other fields such as biology, physics or philosophy have influenced me early on to aim for a research degree and to follow the academic route.

3 ACKNOWLEDGEMENTS

"Focus on the journey, not the destination.

Joy is found not in finishing an activity but in doing it.

Greg Anderson

I want to thank all the great people that supported me and fuelled my enthusiasm. This PhD thesis is the result of their support and success to make me believe in the topic and enjoy the ride.

Professor Gianni Angelini – for sponsoring my PhD tuition fees and for taking ownership of both my academic and clinical career by helping me do my first adult cardiac surgery procedures as the first operator. I am grateful for all his support to get an Academic NTN number in cardiothoracic surgery and for all his great mentorship throughout. He has clearly understood my career objectives and actively supported me throughout.

Professor Stafford Lightman – for giving me the opportunity of working in such a solid, genuine research group but, most importantly, he made me believe in the Peacock Study. To make a cardiac surgical trainee interested in HPA axis physiology and believe in the concept of cortisol pulsatility is a great achievement. The science discussions we had on the top floor in the DHB building were truly inspiring and motivating.

Dr Ben Gibbison – for his thorough and friendly approach. Ben was my first port of call and support for the Peacock Study. His expertise in cortisol pulsatility in adults and accessibility were vital for the supervision of this project.

Dr Francesca Spiga – Fran showed me what it takes to be a solid basic scientist. Her insight into adrenal physiology was precious. The period we worked together for the in vitro project was truly formative and helped me later on to deliver the Peacock Study.

Mr George Horn – for all his training and help with the cells culture experiments and for initiating me into basic cell culture techniques.

Dr Georgina Hazell – for all her help in setting up the THP1 cell cultures and basic cell culture advice.

Dr Jason Johnson – for suggesting the use of THP1 cells in the co-incubation model and for giving me advice on how to culture this cell line.

Dr Thomas Upton – for teaching me the microdialysis insertion technique and general advice throughout the project. Our research meetings held at the "Taste of Napoli" were extremely useful and enjoyable.

Dr Martijn Van Faassen and **Professor Ido Kema**, University of Groningen, Netherlands, for the help with the analysis of the microdialysis cortisol and cortisone samples.

Mrs Terrie Walker-Smith – for showing me how crucial is to have an excellent trial coordinator. She has always delivered what I needed for the project.

Mr Jon Evans for all his support and enthusiasm during the early stages of setting and recruiting for the Peacock Study.

Dr Angela Aramburo, Dr Duncan Macrae. Laura Alacantra – for understanding the importance of Peacock Study and their willingness to recruit patients at Royal Brompton London.

Mr Serban Stoica, Professor Massimo Caputo and Mr Andrew Parry for their patience to take part in the study, ahead of such high-stake surgical procedures. Special thanks to **Mr Serban Stoica** who approached me originally to take part in the Peacock Study and gave me the opportunity to show to my current supervisors what I can do.

For the parents and children that took part in the Peacock study –for the willingness to take part in this research when they had to deal with life-changing procedures.

For my argentine tango maestros – **Alejandro Angelica** and **Ornella Tirrito** – thanks for all the pure tango you taught me and your friendship. This allowed me to switch off at times from the demands of cardiac surgery training and my PhD.

Last but not the least, for the sacrifices of my wife **Andreea** and my two beautiful daughters **Emma** and **Carolina -** thanks for coping with my ambitions and for dealing with my absence. This would not have been possible without your support.

4 AUTHORS DECLARATION

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ..... DATE: .....

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7 ABBREVIATIONS

11β-HSD	11β-hydroxysteroid dehydrogenase
ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of variance
AP-1	Activator protein 1
ATC	Adrenocortical tumour cell lines
ATC7-THP1	ATC7 cells cocultured with THP1 cells
AUC	Area under the curve
AVP	Arginine vasopressin
CBG	Cortisol binding globulin
CCAD	CCAD - Congenital Analysis - Summary Data - By Year
CCAN	Congenital Cardiac Anaesthetic Network
cDNA	Complementary Deoxyribonucleic Acid
CPB	Cardiopulmonary bypass
CREB	Cyclic-AMP response element-binding protein
CRH	Corticotropin-releasing hormone
CS	Corticosteroids
DAMPS	Danger-associated molecular patterns
DAX-1	The dosage-sensitive sex reversal, adrenal hypoplasia congenital critical region on the X chromosome, gene 1
DEX	Dexamethasone
DHCA	deep hypothermic circulatory arrest
DMEM	Dulbecco's Modified Eagle's Medium

FEP	Fluorinated Ethylene Propylene
FSH	Follicle-stimulating hormone
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC-MS	Gas chromatography-mass spectrometry
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
ICU	Intensive care unit
IFN	Interferon
IL-	Interleukin
IRAK	Interleukin-1 receptor (IL-1R) associated kinase
JAK	Janus kinase
KOS	Knife off skin time – end of the operation
KTS	Knife to skin - start of the operation
LC-MS	Liquid chromatography-mass spectrometry
LH	Lutein hormone
LPS	Lipopolysaccharide,
MAPK	Mitogen-activated protein kinase
MC2R	Melanocortin 2 receptor
MP	Methylprednisolone
MR	Mineralocorticoid receptor
MRAP	Melanocortin receptor accessory protein
mRNA	Messenger ribonucleic acid
MUF	Modified ultrafiltration
MyD88	Myeloid differentiation primary response

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PBS	Phosphate-buffered saline
PCREB	Phospho Cyclic-AMP response element-binding protein
PICU	Paediatric intensive care unit
PKA	Protein kinase A
PRRs	Pattern-recognition receptors
PVN	Paraventricular nucleus of the hypothalamus
RACHS	Risk adjustment for congenital heart surgery score
RCT	Randomized controlled trial
REC	Research Ethics Committee
RIP1	Receptor-interacting protein
RNA	Messenger ribonucleic acid
RTqPCR	Reverse transcription-polymerase chain reaction
SCN	Suprachiasmatic nucleus of the hypothalamus
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SF-1	Steroidogenic factor 1 protein
SIRS	Systemic inflammatory response
SPE-LC–MS/MS	Solid-phase extraction-liquid chromatography–tandem mass spectrometry
StAR	Steroidogenic acute regulatory protein
STAT	Signal transducer and activator of transcription
TGF-β	Tissue growth factor β

THP1	Human monocytic cell line derived from an acute monocytic leukaemia patient
TLR4	Tool like Receptor
TNF	Tumour necrosis factor
TRAF	TNF receptor associated factor
ZF	Zona fasciculata

8 CHAPTER 1 INTRODUCTION

''Constancy and stability of the internal environment

is the condition that life should be free and independent''

Claude Bernard

8.1 The stress response – a brief historical note

The concept of biological stress is undoubtedly related to the name of the so-called three "old masters". The first one is the famous physiologist, Claude Bernard (1813-1878) who introduced the concept of the ability of the human organism to maintain a constant internal environment ("milieu interieur") independent of the external challenges (Le Moal 2007; Goldstein and Kopin 2007). Later, Walter Cannon (1871-1945) introduced the concept of "homeostasis" that described the capability of the human body to maintain its function within acceptable physiological ranges. He is also known for the so-called "fight or flight" response and advocated the activation of the sympathetic nervous system to control the adrenal response during stress to restore homeostasis. However, the concept of biological stress was introduced by Hans Selye (1907-1982). He used the term "stress" adapted from the Physics field, where this describes the force necessary to produce a deformity within a material. He advocated the concept of the *nonspecificity* of the stress response and described the three stages of the "General Adaptation Syndrome": the initial alarm phase, the stage of adaptation and the exhaustion phase. In his experimental work, he found a pathological triad of adrenal enlargement, atrophy of the lymphoid tissue in thymus and spleen, and gastrointestinal ulcers as a result of injection animals with a variety tissue extracts or formalin. Later, he demonstrated that these changes are related to the activation of the hypothalamic-pituitary-adrenocortical (HPA) axis (Seyle 1978).

The current, modern, concepts of stress challenge the non-specificity of the stress response and argue that there is a degree of specificity depending on the challenge to the homeostasis. Furthermore, the concept of "allostasis" was proposed to replace "homeostasis"(McEwen 1998). This concept refutes homeostasis, which suggests constancy of values and ranges during stress, and broadly refers to an *adaptative* process to maintain homeostasis. There is now an increased understanding of the impact of psychobiology and psychosocial factors on the mechanism of stress during disease (Le Moal 2007).

My PhD thesis is aimed at understanding the HPA axis physiology by using the stress model of paediatric heart surgery.

8.2 The stress of cardiac surgery

In the third chapter of my thesis, I study the paediatric HPA axis function using the stress model of paediatric heart surgery. Certainly, cardiac surgery is a major stressor of the body. Firstly, anaesthesia itself requires sedation and intubation of the patient. Further stress is added in the anaesthetic room by obtaining intravenous access, arterial access (for invasive blood pressure monitoring) and central venous access. To access the heart, a sternotomy is usually performed that involves the division of the bony chest wall and retraction of the musculoskeletal tissues. Furthermore, to perform open-heart surgery, the patient is attached to the cardiopulmonary by-pass system that is essentially a non-self-extracorporeal circuit that further exacerbates the stress response. At times, the circulation is completely stopped (deep hypothermic circulatory arrest) to perform certain operative steps. The duration of the cardiopulmonary by-pass and the by-pass temperature are other determinants of the magnitude of the stress response. In the postoperative period, the extubation time or other complications such as bleeding or infection can affect the stress reaction. Moreover, significant fluid retention that can occur, particularly

in low weight babies and operations with a long CPB runs, results in haemodilution and pulmonary oedema with ultimately organ dysfunction.

8.3 The systemic inflammatory response to cardiac surgery

Similar to adult cardiac surgery, the combination of anaesthesia, the access to the heart (sternotomy) and use of the CPB evoke a systemic inflammatory response that contributes to the HPA axis activation (Brix-Christensen 2001). According to in vivo studies (Brix-Christensen et al. 2001), the sternotomy itself elicits inflammatory stress; however, the addition of the cardiopulmonary by-pass increases the magnitude of the inflammatory reaction. By extrapolation to adult cardiac surgery, a patient undergoing off-pump coronary artery bypass grafting has a lower inflammatory response than a patient undergoing coronary artery surgery with the use of CPB (Ascione et al. 2000). The extracorporeal circuit contributes to the systemic inflammatory response by (1) immune cell activation in contact with the circuit, (2) mechanical shear stress, (3) haemodilution and (4) hypothermia. Furthermore, this reaction is augmented in children due to the disparity between the surface of the circuit and the surface of the circulatory system. This results in a greater proportion of blood coming into contact with the circuit or cardiotomy suction. Therefore, the systemic activation of immune cells such as polymorphonuclear cells or platelets results in the production of cytokines. The cytokine production is further exacerbated by the ischaemia-reperfusion injury of key organs that are essentially turned off during cardiopulmonary (mainly the heart and the lungs) that results in endothelial activation. Finally, the cardiopulmonary by-pass circuit also results in significant complement activation (C3a, C3d, C5a) that simulates both the endothelial activation and the activation of the immune cells. It is believed that the balance between the production of pro-inflammatory (TNF-α, IL-6, IL-8) and anti-inflammatory cytokines (IL-10 and IL-1 receptor

antagonist) influences the outcomes after cardiac surgery. In addition to these systemic activations, it is believed that a great deal of mortality and morbidity also results from multiorgan dysfunction (heart, lung, kidneys, brain) that results from pooling/accumulation of activated immune cells within these organs (Brix-Christensen 2001; Laffey, Boylan, and Cheng 2002).

8.4 Key receptors and inflammatory cell signalling pathways.

As discussed earlier, the systemic inflammatory response results from cellular activation by the by-pass activations that promote further inflammation by the production of cytokines and chemokines.

One class of receptors that are activated during this systemic inflammation are the germline-encoded pattern-recognition receptors (PRRs) that are expressed in both immune and non-immune cells. One class of PRRs are the Toll-like receptors (TLRs) for examples. Some of PRRs are capable of recognising disrupted or damaged cells called *danger-associated molecular patterns* (DAMPS) in the absence of microbial structures (Chen et al. 2018). Further inflammation signalling also occurs via the various specific cytokine receptors. The major pro-inflammatory cytokines (IL-1, IL-6, TNFα) signal via type I transmembrane cytokine receptors with specific structures (Turner et al. 2014).

Receptor activation triggers activation of three main intracellular signalling pathways: (1) the mitogen-activated protein kinase (MAPK), (2) nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), (3) Janus kinase (JAK)- signal transducer and activator of transcription (STAT) pathways.

The MAPK pathway is activated by various cellular stressors and various cytokines (IL-1, TNF-α, and IL- 6). The NF-κB is activated by TLRs, TNF or IL-1 and regulates the

expression of inflammatory cytokines. The JAK-STAT pathway is activated by IL-6, leading to activation of other cytokines (Chen et al. 2018).

Of importance to the in vitro inflammatory stress model described in the first chapter of my thesis is the LPS/TLR4 signal transduction pathway (Figure 1). Briefly, the LPS is an essential structure of gram-negative bacterial all. As discussed earlier, the TLRs are expressed by cells of the innate immune systems that are capable of recognising structural motifs expressed by bacteria, viruses or fungi called pathogen-associated molecular patterns (PAMPs).

LPS activation of various mammalian cells occurs via complex extracellular and intracellular interactions. Firstly, the LPS binds to the LPS binding protein (LBP), a shuttle protein, that facilitates the associated between the LPS and cluster of differentiation 14 (CD14). CD-14 is a glycosylphosphatidylinositol-anchored protein that helps the transfer of the LPS to the TLR4/MD-2 receptor complex and facilitates its recognition. Downstream, the TLR4 signalling pathway occurs via a myeloid differentiation primary response 88 (MyD88)-dependent and MyD88-independent pathways [TIR-domain-containing adapter-inducing interferon-β (TRIF) dependent]. The MyD88 dependent pathway activates the interleukin-1 receptor (IL-1R) associated kinase (IRAK) (IRAKs)/ TNF receptor-associated factor (TRAF) 6 as well as the transcription factors NF-κB, activator protein 1 (AP-1) and interferon regulatory factor 5 (IRF-5) further downstream and induce the expression of pro-inflammatory cytokine genes. The MyD88 independent pathway involves signalling via Type I interferons that recruit TRAF3 and receptor-interacting protein 1 (RIP1) to activate transcription factor IRF3, as well as NF-κB and AP-1 (Lu, Yeh, and Ohashi 2008).

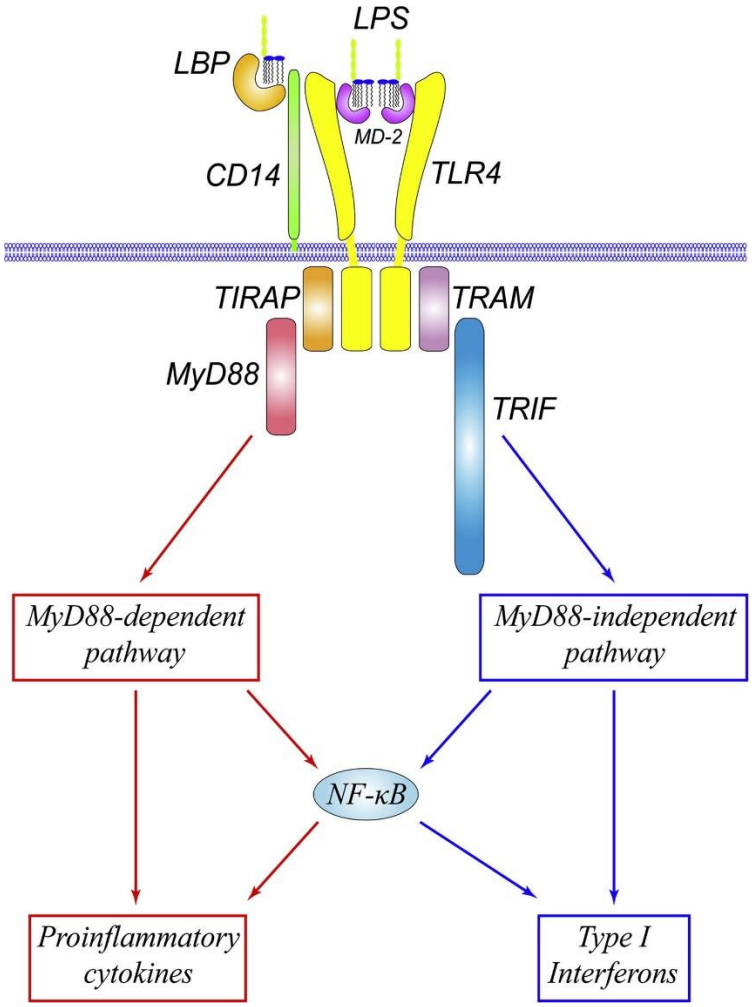

Figure 1 LPS/TLR4 signalling pathway (From. Lu, Yeh, and Ohashi 2008)

8.5 The HPA axis: basic control mechanisms

The human body's acute stress response comprises sympathetic nervous system activation, an endocrine response and an immunological and haematological reaction (Desborough 2000). The hypothalamic-pituitary-adrenal (HPA) axis regulates the primary neuroendocrine response that is activated to produce glucocorticoids (corticosterone in rodents and cortisol in humans) for adequate homeostatic regulation (Spiga et al. 2014). The stressors are integrated by the brain stem and limbic areas, and inputs to the hypothalamic paraventricular nucleus are thus generated. The paraventricular nucleus (PVN) projects to the median eminence where corticotropin realising hormone is produced. The corticotropin-releasing hormone (CRH) then reaches the anterior pituitary via the portal circulation to activate the adrenocorticotrophic hormone (ACTH) secretion in the venous circulation. The ACTH activates both the production and release of cortisol from the *zona fasciculata* of the adrenal gland cortex (Spiga et al. 2014; B. Gibbison, Angelini, and Lightman 2013) (Figure 2). Within the HPA axis, there is a negative feedback control exerted by cortisol at the pituitary and hypothalamic level. Therefore, cortisol regulates its own production by inhibition of the production and release of the ACTH from the anterior pituitary and inhibition of the CRH by direct action on the PVN and other hippocampal structures that modulate the PVN (amygdala and prefrontal cortex) (Spiga et al. 2015).

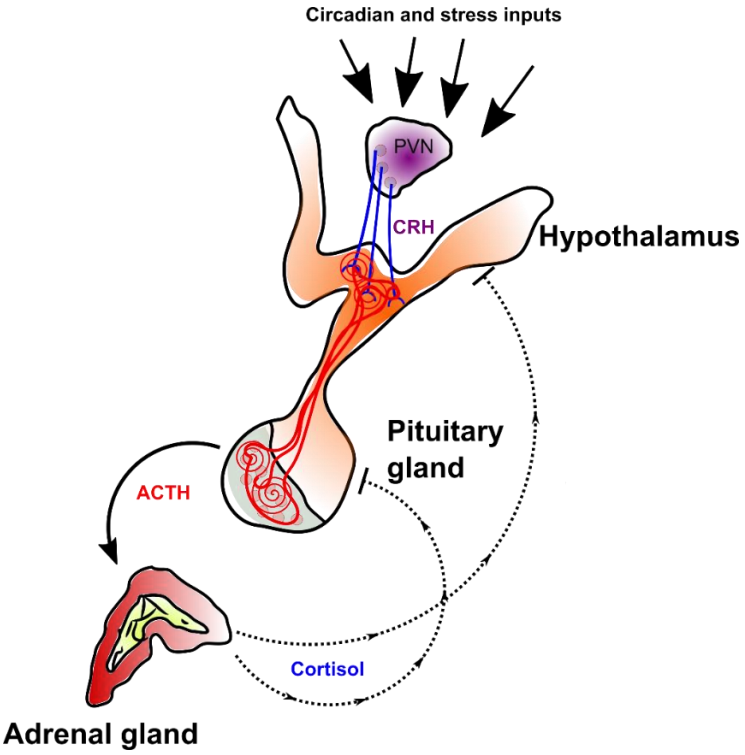

Figure 2. HPA axis control

8.6 Adrenal steroidogenesis

Due to their lipophilic nature, glucocorticoids cannot be stored in granules and need to be synthesised *de novo* in the adrenal cortex in response to ACTH stimulation (Figure 3). Binding of ACTH to its specific receptor, the melanocortin type-2 receptor (MC2R) leads to activation of the protein kinase A (PKA) pathway, which, in turn, results in the secretion of glucocorticoid as well as steroidogenic gene expression, via *non-genomic* and *genomic* pathway, respectively (reviewed in (Miller and Auchus 2011)). While the *non-genomic pathway* includes the phosphorylation, thus activation, of steroidogenic proteins including the rate-limiting protein steroidogenic acute regulatory protein (StAR) (Arakane et al. 1997; Stocco and Clark 1996; Strauss et al. 1999), the genomic pathway regulates the transcription of steroidogenic proteins and its transcriptional regulators. This includes transcription of the steroidogenic proteins such as StAR, MC2R, melanocortin receptor accessory protein (MRAP,

a protein that regulates MC2R expression (Metherell et al. 2005) as well as the orphan nuclear receptor – steroidogenic factor (SF-1) (Sugawara et al. 1996) and the transcriptional inhibitor (the dosage-sensitive sex reversal, adrenal hypoplasia congenital critical region on the X chromosome, gene 1) DAX-1 (Zazopoulos et al. 1997; Iyer and McCabe 2004) (Figure 3). This brief overview is necessary because in Chapter 2 of the thesis I will explore the effect of LPS stimulation on both the *inflammatory pathway* and *steroidogenic pathway* in isolated adrenal cells or adrenal cells co-incubated with immune cells.

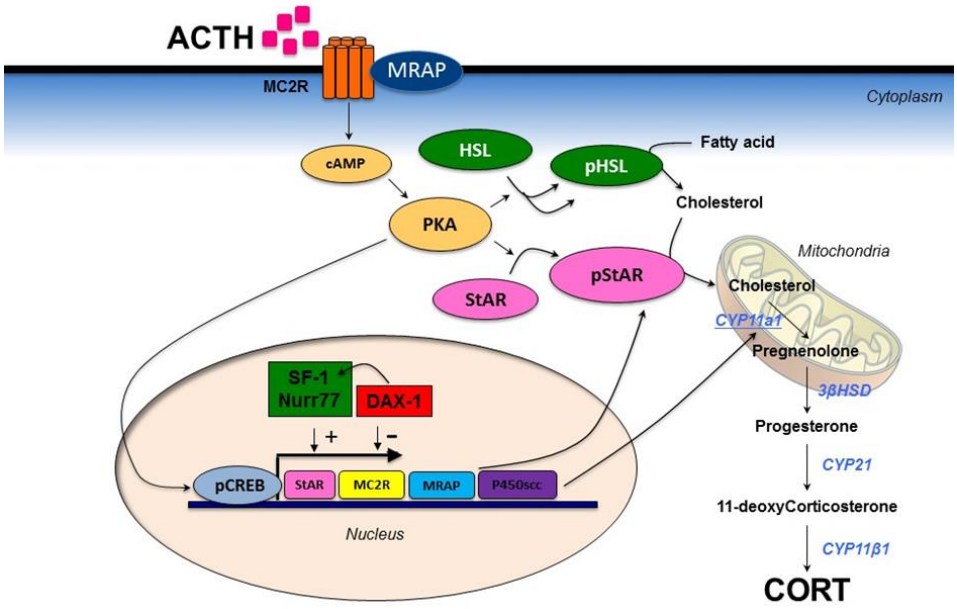

Figure 3 Adrenal Steroidogenesis (Adapted from F. Spiga et al.)

8.7 The glucocorticoid receptor and its development

Glucocorticoids exert their physiological and pharmacological effects by acting on the glucocorticoid receptor. The glucocorticoid receptor (GR) is a member of the nuclear receptor superfamily of ligand-dependant transcription factors (Oakley and Cidlowski 2013). Due to its lyophilic nature, cortisol traverses the cell membrane and binds to the GR complex. This complex is made up of the GR receptor in combination with heat shock protein 90 and p23 that facilitate binding to cortisol. After cortisol binding, these stabilising proteins dissociate and are

recycled while the Cortisol-GR complex dimerises and is to be transported in the nucleus via nuclear pore special complex. At the nuclear level, the activated GR binds to the glucocorticoid responsive elements (GRE) of the DNA to control transcription. In some cells, the membrane-bound GR (mGR) exerts its effects via non-genomic pathway (Figure 4) (B. Gibbison, Angelini, and Lightman 2013).

The GR receptor is expressed in almost all tissues. In adults, the GR effect can vary considerably in the different tissues of and an individual (Oakley and Cidlowski 2013). A pertinent question to my thesis is how the GR receptor expression develops by age, and how does this affect the physiological and pharmacological actions of steroids give to children of various ages?

The development of the GR receptor has been studied extensively in the brain. In vitro studies by Meaney et al. (Meaney, Sapolsky, and McEwen 1985) have shown low levels of the GR receptor in brain rat cells perinatally with an increase in the levels at the end of the first week of life that paralleled the increase of the circulating corticosterone. This suggests a lack of GR autoregulation that is seen in adults where increases in corticosterone levels result in a decrease in the GR receptor.

Glucocorticoids have a significant role in the development of several tissues apart from the brain such as the lung, intestine, neural retina or the HPA axis. It has been shown that the GR receptor is present in the above tissues in the fetus but only at a minimal level. However, there is a general agreement that adult GR receptor levels are reached between 5 and 15 postnatal days (Kalimi 1984). Therefore, we to keep in mind that the differential expression of GR by age can affect glucocorticoid responsiveness.

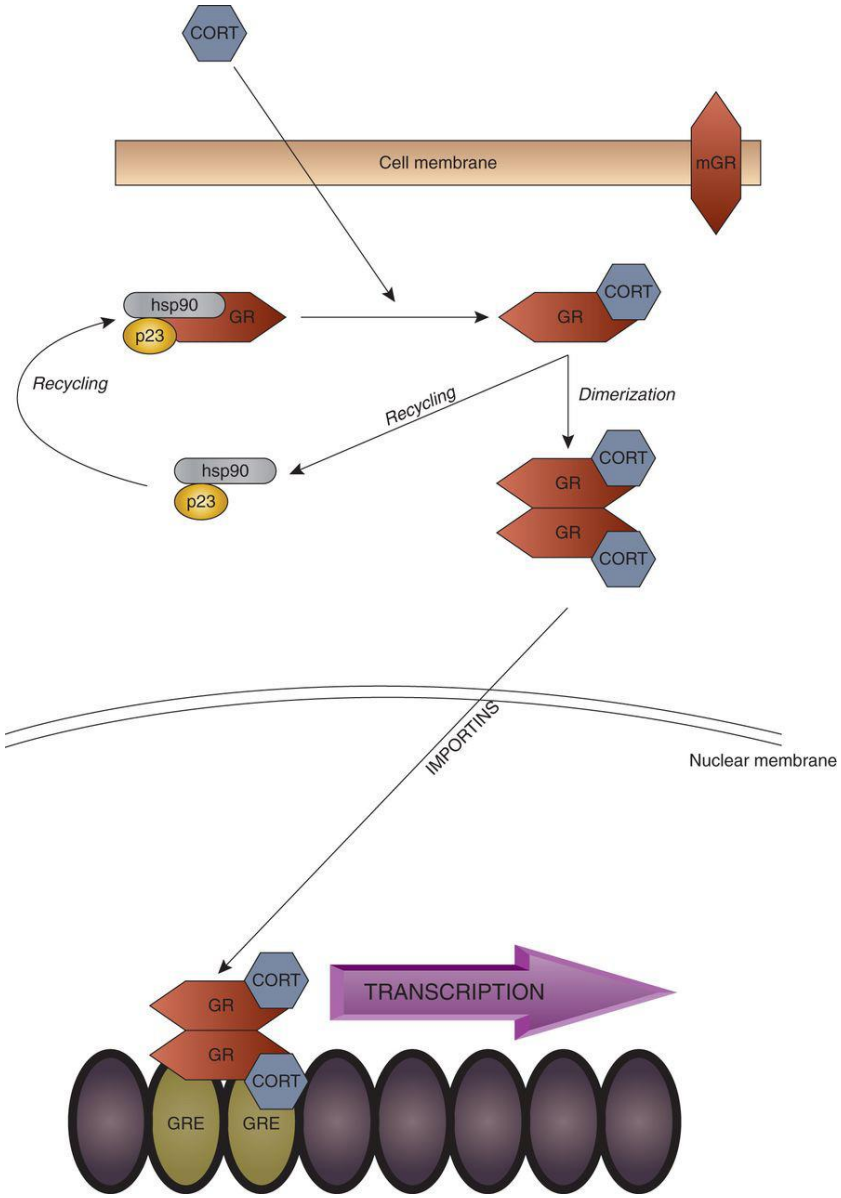

Figure 4 Glucocorticoid signal transduction via GR (From (B. Gibbison, Angelini, and Lightman 2013)

8.8 1.1 Cortisol regulation at the tissue level: the role of the 11 β-hydroxysteroid dehydrogenases

Apart from the regulation of the glucocorticoid actions in the target tissues by the density of GR receptor, an important role is played by the intracellular metabolism of cortisol at tissue level (Chapman, Holmes, and Seckl 2013). The crucial regulators are the two isoenzymes of the 11β-hydroxysteroid dehydrogenase (11β-HSD) (Figure 5).

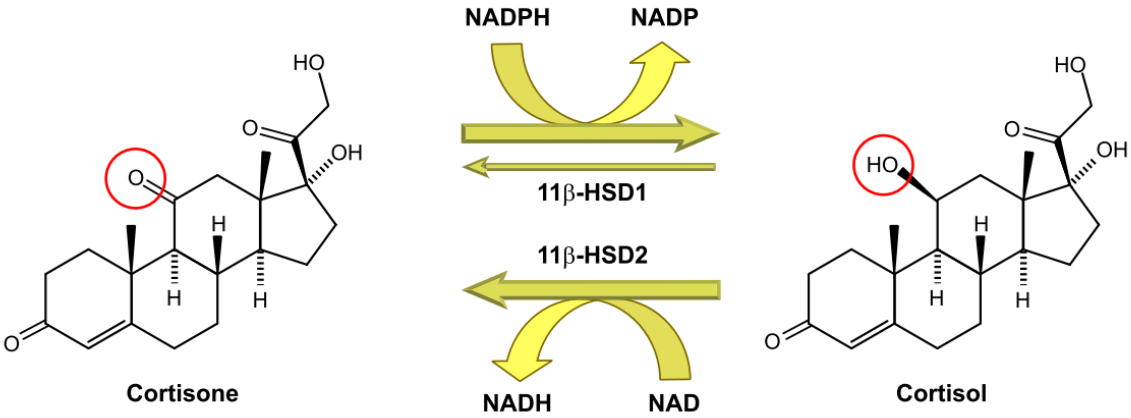

Figure 5 The 11β-hydroxysteroid dehydrogenase type 1 and 2 action (from Chapman et al. 2013)

The 11β-HSD1 is a reductase that catalyses the regeneration of active cortisol. It is widely expressed in liver, adipose tissue, muscle, pancreatic islets, adult brain, inflammatory cells (mainly macrophages), and gonads. The expression is highest in the liver. Interestingly the 11β-HSD1 is expressed late in gestation mainly in liver and lung where the glucocorticoid action is required for maturation of these organs. In the context of inflammation/immunity, the 11β-HSD1 is highly expressed in macrophages activated by LPS as compared to the monocytes (undifferentiated cells) where levels are quite low (Chapman, Holmes, and Seckl 2013). Hence, in the context of systemic activation of the immune cells, the 11β-HSD1 can exert a pro-inflammatory effect.

The 11 β-HSD2 is a dehydrogenase that inactivates the cortisol to inert cortisone. In contrast to 11β-HSD1, the 11 β-HSD2 is not expressed in immune cells except in some human rheumatological conditions such as rheumatoid arthritis. The expression of the 11 β-HSD2 in the kidney, inactivates cortisol to cortisone and ensures that the only agonist of the MR receptor is aldosterone (see the relevance in the Discussion chapter 11.6.14). The 11 β-HSD2 is highly expressed in the fetal and placental tissues where the glucocorticoid inactivation essentially prevents the premature maturation of the fetal tissues and protects against developmental programming[1] that can be induced by maternal stress.

[1] During foetal development the environmental influences can shape the developing organism structure and function in such a manner that these effects persists for the lifespan. In the context of the maternal stress model discussed above, early exposure of the foetus to maternal glucocorticoids can influence the prevalence of cardiac, metabolic and psychiatric disorders during later life (Del Giudice 2012; Barker 2004) .

8.9 HPA axis rhythms

HPA axis pulsatility is present in various species studied to date, including the: rat, monkey, hamsters, horse, sheep, goat and human (Stafford L. Lightman et al. 2008). The secretion of ACTH and cortisol display a dynamic, circadian pattern. Underlying the circadian rhythm, there is an *ultradian rhythm* of fine pulses (Figure 6). Studies by our group have demonstrated that the feedforward - feedback in the pituitary-adrenal system is enough to produce the oscillations of the ultradian rhythm. (Walker, Terry, and Lightman 2010).

Further forcing by the SCN above superimposes the circadian rhythm on top of this. Thus, the highs of the circadian rhythm are produced by large-amplitude pulses and the lows by little or no pulsatility. Hormone pulsatility is necessary for accurate regulation of target genes by the glucocorticoid receptor (GR) (Conway-Campbell et al. 2010; Stavreva et al. 2009). It has been shown that circadian and ultradian rhythms show variation according to sex (Seale et al. 2004), genetic background (R. J. Windle et al. 1998), age (S L Lightman et al. 2000) and reproductive cycle (Richard J. Windle et al. 2013). Furthermore, rats exposed to early stress during neonatal period display a change in their pulsatility in adult life, a phenomenon called *neonatal programming* (Shanks et al. 2000). During disease, changes in pulsatility were demonstrated in models of chronic stress in the rat (R. J. Windle et al. 2001; Waite et al. 2012) and during cardiac surgery in human (Ben Gibbison et al. 2015).

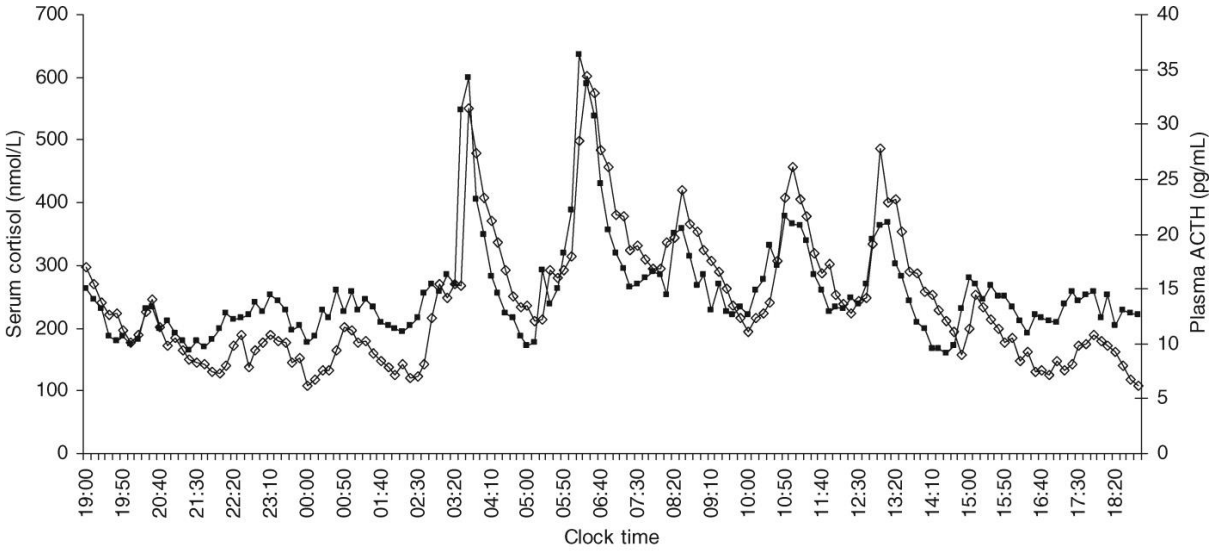

Figure 6 Profiles of serum cortisol concentrations (nmol/L) in a healthy volunteer. Rapid (10-minute) automated sampling demonstrates the circadian profile and the underlying ultradian rhythm (from Henley, D. E., Leendertz, J. A., Russell, G. M., Wood, S. A., Taheri, S., Woltersdorf, W. W., & Lightman, S. L. (2009). Development of an automated blood sampling system for use in humans. Journal of Medical Engineering and Technology, 33(3), 199–208. https://doi.org/10.1080/03091900802185970)

8.10 The ACTH-Cortisol Dissociation

According to this "traditional" model, any increase in ACTH secretion to acute stress would result in concomitant increase cortisol. However, during surgery and critical illness, a so-called "ACTH-cortisol dissociation" occurs (Boonen, Bornstein, and Van den Berghe 2015). After the stress stimulus (surgery) there is an initial rise in ACTH followed by cortisol increase, then, the ACTH falls, but the cortisol remains elevated. This finding is a matter of debate and can be explained by several mechanisms (Figure 7) including altered cortisol metabolism (Boonen et al. 2013), saturation of CBG (Roth-Isigkeit, Dibbelt, and Schmucker 2000; Wald et al. 2011b), splanchnic nerve activity (Ulrich-Lai, Arnhold, and Engeland 2006) increased sensitivity of the adrenal cortex to ACTH (Ben Gibbison et al. 2015) and local, adrenal "tissue" mechanisms (Boonen, Bornstein, and Van den Berghe 2015).

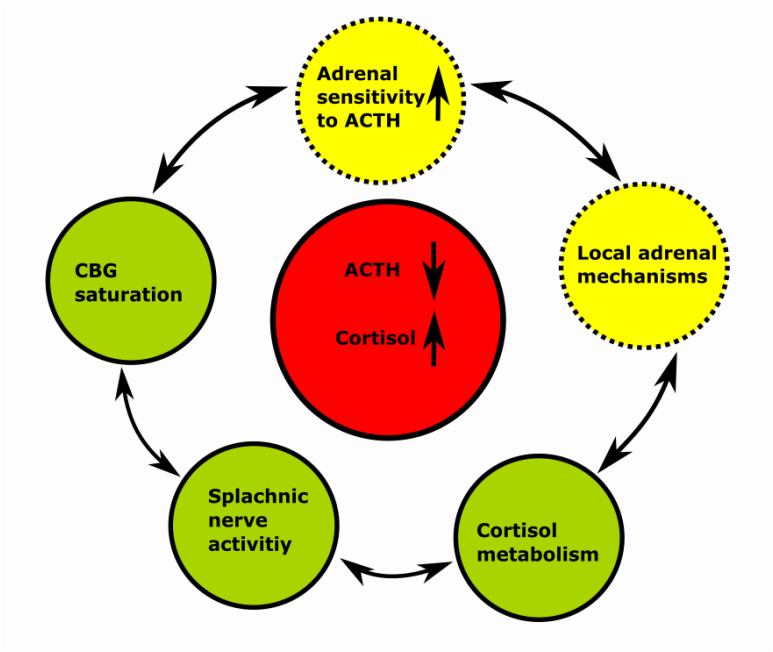

Figure 7 ACTH-Cortisol dissociation factors

To support the concept of an adrenal, local inflammatory mechanism responsible for this so-called dissociation, the systemic intravenous injection of LPS in the rat reproduces the ACTH-cortisol dissociation observed in adults undergoing heart surgery. This is opposed to the finding that an ACTH subcutaneous injection in the rat results in a return of both cortisol and ACTH to basal (Ben Gibbison et al. 2015).

8.11 Cortisol binding globulin

Cortisol is relatively insoluble in plasma; hence, most of the hormone in the plasma is bound to its main protein carrier - cortisol binding globulin (CBG). CBG is mainly produced in the liver but also in the lung, kidney and testis (Hammond et al. 1990). The binding capacity of CBG is finite, and in adults, it occurs at cortisol levels of approximately 400-500 nmol per litre (Ballard 1979).

Cortisol levels that exceed this threshold result in a disproportionate amount of free cortisol and any pulses that exceed this level lead to large swings of free active cortisol. Furthermore, the CBG's affinity to cortisol can be affected by temperature (Cameron et al. 2010; D.E. Henley and Lightman 2011) and neutrophil activity (Hammond et al. 1990). Granulocytes from septic patients incubated with CBG destroy the steroid-binding activity (Hammond et al. 1990), and fever or external temperatures releases cortisol from CBG (Cameron et al. 2010; D.E. Henley and Lightman 2011). All these findings suggest that cortisol delivery can be increased in areas of inflammation because of reduced affinity for CBG. Cameron et al. used recombinant human CBG to assess the binding to cortisol depending on temperature and pH. At 37 C degrees for example, the fall of pH from 7.4 to 6.8 does not modify the binding affinity of cortisol to CBG however it does affect the binding of cortisol to other protein such as albumin (Cameron et al. 2010; D.E. Henley and Lightman 2011).Therefore, CBG is both a reservoir and regulator of cortisol (B. Gibbison, Angelini, and Lightman 2013).

8.12 Cortisol pulsatility during adult cardiac surgery – Gibbison et al. studies

Gibbison et al. (Ben Gibbison et al. 2015) characterised the pulsatile nature of ACTH and cortisol during and after cardiac surgery (coronary artery by-pass grafting - CABG) in adults by using blood measurements every 10 minutes. Then, the authors used a translational rat model where they have replicated the same ACTH/cortisol pattern after LPS injection and looked at the changes in the adrenal steroidogenic pathway. The main findings of the study were (1) maintenance of ACTH-cortisol ultradian pulsatility across the entire peri-operative period and (2) increased adrenal sensitivity to ACTH (Figure 8). This maintenance of ACTH-cortisol pulsatility is contrary to previous studies that advocate a dissociation (Vermes et al. 1995; Mackie et al. 2011; Roth-Isigkeit, Dibbelt, and Schmucker 2000).

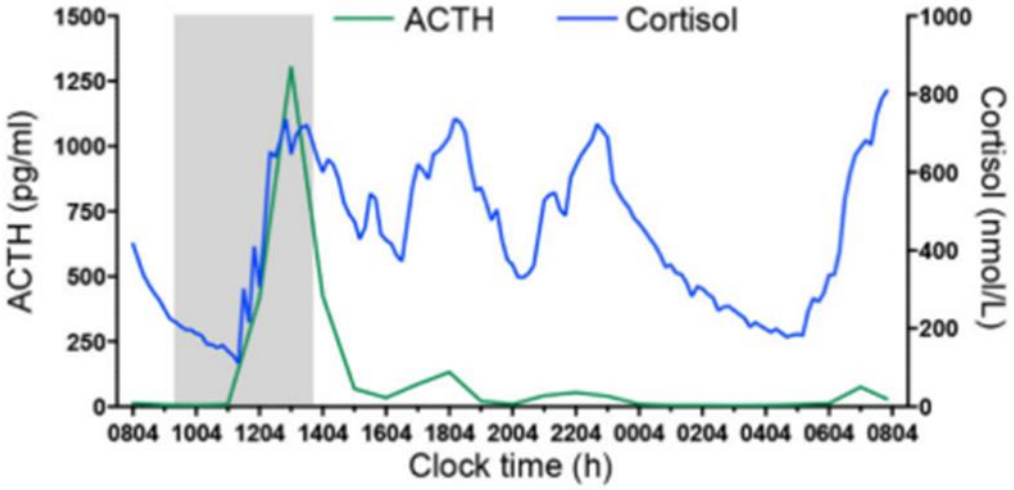

Figure 8 Twenty-four-hour cortisol profile of a patient undergoing CABG (from Gibbison et al.)

8.13 Justification of my three lines of research

This PhD thesis is built around three main lines of investigation (Figure 9). The first direction was to try to understand the local adrenal mechanisms responsible for the ACTH-cortisol dissociation. This led us to develop a novel co-incubation model to explore the

immune-adrenal cross talk (Chapter II). Building from the adult cortisol studies, the second line of research was aimed at understanding the basic HPA axis response in children undergoing heart surgery (Chapter III and IV). Why study the HPA axis function in children? The interest in this area of research resulted from how little we know about the paediatric stress response. This limited understanding of paediatric HPA axis physiology is undoubtedly reflected in a lack of consensus around the use of synthetic glucocorticoids administered perioperatively. Therefore, a significant part of my PhD was dedicated to understanding the evidence for giving glucocorticoids perioperatively (Chapter III). Finally, I focused on trying to understand the basic stress response in children by using a novel approach that allows recording the dynamic HPA function during and after surgery (The Peacock Study) (Part IV).

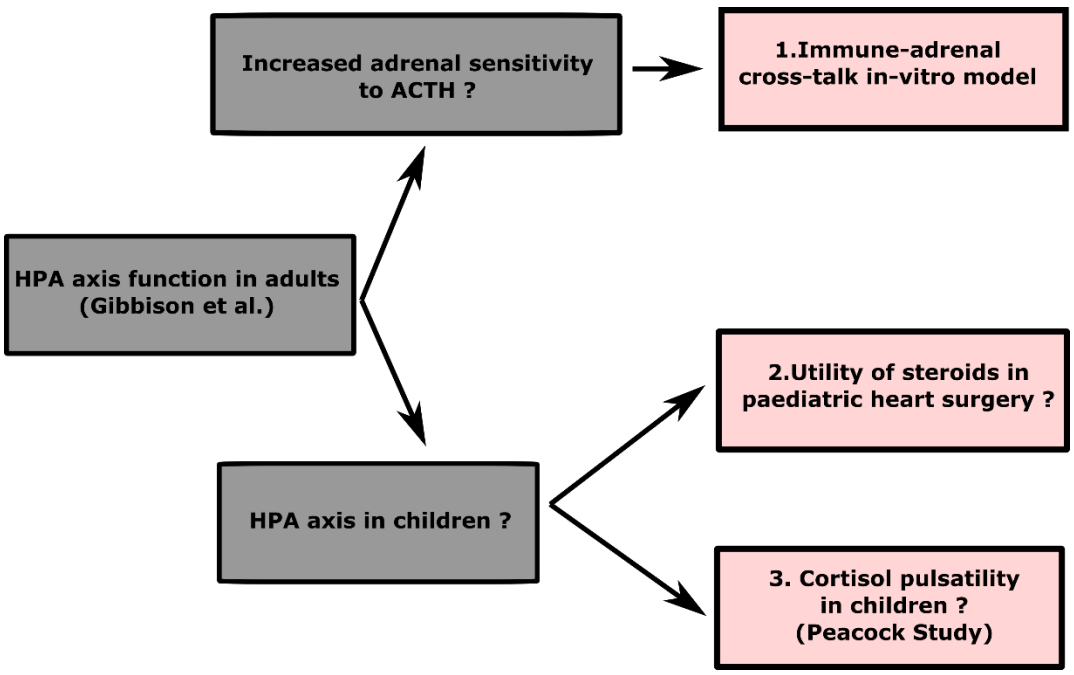

Figure 9 Research directions of my thesis

9 CHAPTER 2 THE ROLE OF THE IMMUNE-ADRENAL CROSS TALK DURING INFLAMMATORY STRESS

"The body is a community made up of its innumerable cells or inhabitants."

Thomas A Edison

9.1 Introduction

9.1.1 The immune-adrenal crosstalk

As discussed in chapter 1, one of the aims of my research was to understand how the local adrenal mechanisms affect intraadrenal cytokine expression and the activation of the steroidogenic pathway during inflammatory stress. As seen later, I focus initially on understanding the response of isolated on adrenal cells to inflammatory stress (LPS stimulation) and then I further hypothesise that an immune-adrenal cross talk can occur. By using a co-culture model, I explore the cellular interaction between the adrenal cells and the surrounding immune cells (Figure 10). This cross-talk can occur via cytokines produced by adrenal cells themselves or by the neighbouring immune cells to regulate steroidogenesis (Bornstein, Rutkowski, and Vrezas 2004). The above hypotheses are supported by several studies. In an *in vivo* study in rats, lipopolysaccharide infusion induced systemic inflammation that was accompanied by infiltration of leukocytes in the adrenal gland (Kanczkowski, Chatzigeorgiou, et al. 2013). In a model of sepsis, induced by caecal ligation and puncture in mice, the non-survivors had a significant increase of interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumour necrosis factor-α (TNFα) in adrenal protein extracts (Jennewein et al. 2016). It is also possible that the local modulation of the adrenocortical cell function can occur directly, for example, via the toll-like receptors that recognise external immune insults

including lipopolysaccharide (LPS), and that are expressed in adrenocortical cells (Bornstein et al. 2004), or via circulating cytokines activating the adrenal cytokine receptors. However, it remains unclear if the plasma level of immune-derived cytokines is high enough to regulate directly the adrenocortical steroidogenesis or if they have to be locally, intra-adrenally secreted (Ehrhart-Bornstein et al. 1998).

Nevertheless, the adrenal cells produce a variety of cytokines such as IL-1, interferon-gamma inducing factor (IGIF), IL-6 and TNFα. Moreover, steroidogenesis has been shown to be influenced directly by IL-1α, IL-1β, interleukin IL-2, IL-6, TNFα, interferon-alpha (IFNα) in several *in vitro* experiments (Ehrhart-Bornstein et al. 1998). A previous study using primary cultures of human adrenocortical cells co-cultured with human monocytes has shown a significant increase in cortisol production by the adrenal cells. In this study, the monocyte induced cortisol increase was much higher compared to the direct IL-1 stimulation (Whitcomb et al. 1988).

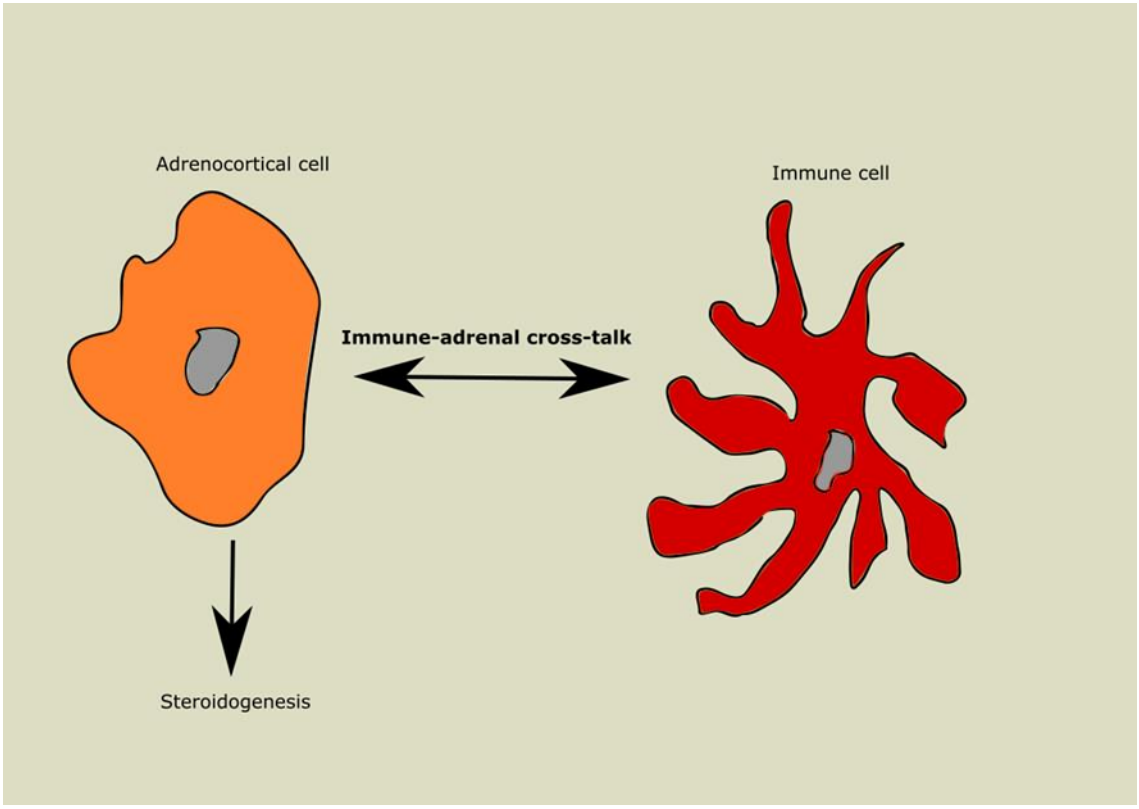

Figure 10 Immune-adrenal cross talks

9.1.2 Development of the ATC7-THP1 cells model

During my *in-vitro* project, a great deal of effort was invested in trying to obtain a significant expression of pro-inflammatory cytokines in immortalised adrenal cells (adrenocortical tumour cell lines - ATC cells). This lack of success made me understand that the adrenal response might be dependent on the presence and activation of immune cells. Therefore, the current thesis describes a novel co-incubation model of ATC with complete *zona fasciculata* (ZF) cell phenotype (Ragazzon et al. 2006; Hazell et al. 2019) and macrophage-derived from THP1 monocytes (a human monocytic cell line derived from an acute monocytic leukaemia patient) (Tsuchiya et al. 1980). Using this model, I explored the effects of an immunological stimulus (lipopolysaccharide, LPS) on the expression of the pro-inflammatory cytokine IL-6, as well as the expression of genes involved in steroidogenesis in ATC7 cells, both in basal conditions and under ACTH stimulation. Synthetic glucocorticoids are still widely used in clinical practice to modulate the immune and adrenal response to acute stress observed during sepsis or surgery, however, their use and mechanism of action remain a matter of intense debate in this setting (D. P. Fudulu, Schadenberg, et al. 2018; D. P. Fudulu, Gibbison, et al. 2018; D. P. Fudulu, Lightman, et al. 2018). Therefore, in this study, I also investigated the temporal effects of glucocorticoid treatment on ATC7 cells responses to LPS (see the section below).

9.1.3 The bimodal effect of glucocorticoids

Glucocorticoids are well known for their anti-inflammatory effects, but less well known are their possible proinflammatory effects initially suggested by Ingle in 1952 and Selye in 1954 (Yeager et al. 2016) have been largely ignored. Münck revisited this concept in 1984.

Since the ‘80s a bimodal, permissive-suppressive model (Figure 11) is being researched and advocated by several groups (Yeager, Guyre, and Munck 2004; Yeager et al. 2016; 2009; Sapolsky, Romero, and Munck 2000). These antagonistic effects seem to be influenced by the temporal relation between the inflammatory insult and glucocorticoid exposure. According to this model, at basal concentrations, in the absence of a stressor, glucocorticoids are permissive by supporting the metabolic and inflammatory processes. The absence of this activity leads to the well-known Addisonian crisis. However, when glucocorticoids are present in higher concentration, in the context of concurrent exposure to stress stimulus, they exert an anti-inflammatory action by preventing the damaging effect of excessive inflammation. Interestingly, when there is exposure to a high concentration of glucocorticoids that is followed by no stimulation and return of glucocorticoid levels to normal, preparatory effects are noticed. These changes are phenotypic and transcriptional and were noted in immunocompetent cells (Yeager et al. 2016). Subsequent exposure to inflammatory exposure results in a pro-inflammatory effect that is meant to increase the organism resistance to subsequent injury.

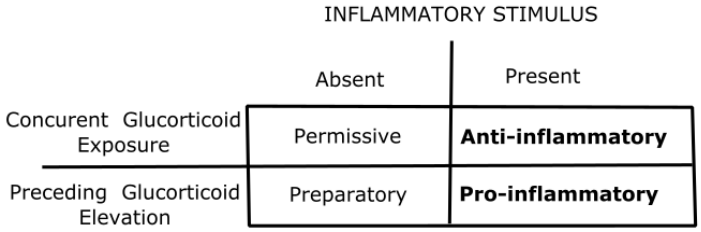

Figure 11 Pro-anti-inflammatory model (adapted from Yeager et al. 2016)

For example, Johnson et al. (Johnson et al. 2002) showed that prior exposure of rats to inescapable tail shock followed by LPS injection resulted in a more rapid and pronounced expression of the inflammatory cytokines compared to the non-stressed rats and this phenomenon was demonstrated to be GR dependent since adrenalectomy or GR receptor antagonists (RU486) abolishes it (Horowitz and Zunszain 2015).

9.2 Hypothesis and aims

The starting point of this work was to explore the local adrenal mechanisms responsible for the increased adrenal sensitivity to ACTH demonstrated by Gibbison et al. (Ben Gibbison et al. 2015) The initial focus was to use isolated adrenal cells (ATC7 cells) that were exposed to inflammatory stress to study the expression of adrenal inflammatory cytokines and the impact on steroidogenesis. Besides, I have also tried to test if glucocorticoids have a bimodal effect (e.g. pro- and anti-inflammatory) on isolated adrenal cells. However, despite much effort, I could not induce a significant expression of inflammatory cytokines after LPS stimulation within the isolated adrenal cells. Therefore, the central hypothesis shifted towards testing if the expression of the adrenal cytokines is dependent on the presence of immune-competent cells. This required the development of a co-incubation model of adrenal cells and immune cells. Using this novel model, I investigated the effect of LPS, glucocorticoids and ACTH on the expression of adrenal cytokines (IL-6 mRNA and the changes in the key steroidogenic genes.

In this chapter, the following hypotheses have been tested:

1) Stimulation of isolated adrenal cells (ATC7 cells) with LPS induces the expression of the pro-inflammatory cytokines IL-6 mRNA;
2) The presence of immune cells (THP1 cells) influences the LPS induced pro-inflammatory cytokines IL-6 mRNA and steroidogenic gene expression;
3) Glucocorticoids exert a bi-modal effect on the expression of adrenal IL-6 mRNA in the ATC7 cells co-cultured with THP1 cells;

4) The ACTH induced adrenal IL-6 mRNA expression, and the steroidogenic pathway is influenced by the temporal administration of LPS and glucocorticoids (pre- or co-treatment)

Furthermore, the aims of this chapter are shown as following:

1) To test the effect LPS on IL-6 mRNA expression in the isolated ATC cells (ATC1 and ATC7 cells);
2) The determine the optimal co-incubation ratio of ATC7 cells and THP1 cells;
3) To determine the optimal LPS stimulation dose pf ATC7-THP1 co-culture;
4) To assess the time course of LPS incubation on IL-6 mRNA expression and steroidogenic gene expression.
5) To assess the effects of co-incubation with the synthetic glucocorticoid dexamethasone (DEX) on LPS induced IL6-mRNA expression.
6) To investigate the effect of DEX pre-treatment on LPS induced IL6-mRNA expression.
7) To investigate the effect of LPS on ACTH- induced IL-6 mRNA and steroidogenic gene expression in ATC7 and ATC-THP1 cells.

9.3 Methods

9.3.1 Single-cell type culture, trans-well co-culture and cell treatments

Murine adrenocortical tumour ATC7 cells (from Dr Pierre Val, Université Clermont Auvergne, Clermont-Ferrand, France), were cultured on poly-L-lysine-coated 75cm^2 tissue culture flasks in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 medium (DMEM-F12) at 37°C in a 5%CO2-95% air atmosphere. The medium was supplemented with insulin (10 mg/ml), transferrin (5.5 mg/ml), and selenium (5 ng/ml) (ITS), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), 2.5% horse serum, and 2.5% fetal bovine serum. Cells were passaged every 3-4 days, and the culture medium changed every 2-3 days. For all the experiments, cells were seeded 2-3 days before experimentation into poly-L-lysine-coated 6-well plates at 5-7 x105 cells/well. Human monocytic THP1 cells (Sigma, Gillingham, UK) were cultured in suspension in 75cm^2 tissue-culture flasks in DMEM at 37°C in a 5%CO2-95% air atmosphere. The medium was supplemented with 20% horse serum penicillin (100U/ml) and streptomycin (100ug/ml). Cells were passaged every 3-4 days, and culture media changed every 2 days. Differentiation of THP1 cells was achieved by resuspending THP1 cells in medium containing 100nM PMA (Sigma) in 6 wells plate polycarbonate cell culture inserts (TC inserts, Sarsted, Nümbrecht, Germany). Cells were left to differentiate for 72 hours then washed twice with 1x PBS (phosphate-buffered saline, pH 7.4, ThermoFisher, Waltham, MA USA). The insert containing THP1 cells was then transferred into a six wells plate containing ATC7 cells and incubated in serum-free media (DMEM/F12/0.1% BSA). The ratio ATC7:THP1 was kept at 1:2 for all experiments except on the ratio experiment in which different ratio ATC7:THP1 were tested. Both ATC7 and THP1 cells were serum-starved in serum-free medium supplemented with 0.1% BSA approximately

16-24 hours before the start of each experiment. ATC7 or ATC7+THP1 cells were incubated with: LPS (Lipopolysaccharides from Escherichia coli O111:B4; Sigma, UK), proinflammatory cytokines (mouse IL-1β, IL-6 and TNFα, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), dexamethasone (DEX, Dexamethasone 21-phosphate disodium salt; Sigma); ACTH (adrenocorticotropic hormone from porcine pituitary, Fragment 1-39; Sigma) as described in detail for each experiment in the result section. At the end of each experiment, cells were washed with ice-cold phosphate-buffered saline (PBS), and then sodium dodecyl sulfate (SDS)-lysis buffer (2% SDS, 50mM Tris pH 6.8, 10% glycerol) was added to each well. Cells were scraped off, and the lysate was collected in two aliquots and stored at -20C until processing for RNA and protein extraction as described below.

9.3.2 Quantitative RT-PCR

For RNA quantification, cells were lysed in RNA lysis buffer, and total RNA was purified using Ambion Pure-Link kit (Invitrogen, ThermoFisher Scientific). The cDNA template was reverse transcribed from 1000ng of total RNA using Cloned AMV First-Strand cDNA synthesis kit (Invitrogen, ThermoFisher Scientific). RTqPCR was performed using Power SYBR green PCR mix (Applied Biosystems, ThermoFisher Scientific) and a 4 ng cDNA template. Samples were amplified by an initial denaturation at 50 °C for 2 min, 95 °C for 10 min and then cycled (40-50 times) using 95 °C for 15 s and 60 °C for 1 min. Genes were normalised to GAPDH mRNA as determined in a separate real-time PCR reaction. RTqPCR primers (Table 1) were used at a final concentration of 200nM and designed to span an exonic-exonic region to detect mature transcript (mRNA).

Gene target	Forward	Reverse
GAPDH mRNA	CCATCACTGCCACCCAGAAGA	GACACATTGGGGGTAGGAACA
IL6 mRNA	GCCTTCTTGGGACTGATGCT	GCCATTGCACAACTCTTTTCTCA
StAR mRNA	TCGTGAGCGTGCGCTGTACC	CTTCGGCAGCCACCCCTTCAG
MC2R mRNA	CCAAGGCCCTTCTAAGCCAG	CTTGCGGTGTCATTGGTGTG
MRAP mRNA	AGTCATGGCCAACGGGACCG	GGGACTGTGCCTCATCTGTGGGG
SF-1 mRNA	AGGAGGAAAGGACGATCGGA	ACCTTGTCACCACACATGG
DAX-1 mRNA	ACCGTGCTCTTTAACCCAGA	CCGGATGTGCTCAGTAAGG

Table 1 RTqPCR primers sequence

9.3.3 Western immunoblotting

For protein quantification, cells were lysed in SDS lysis buffer (2% SDS; 50 mM Tris pH 6.8; 10% glycerol) and Western immunoblotting performed as described in Hazell et al. (Hazell et al. 2019) In brief, all membranes were blocked with 1% BSA in Tris-buffered saline/0.05% Tween 20 (TBS/T) and probed with primary rabbit antibodies directed to StAR (1:1000; Santa Cruz Biotechnology, USA), pCREB (1:1000; Cell Signalling Technology, Inc., USA), followed by horseradish peroxidase-conjugated donkey α-rabbit secondary antibody (1:5,000; Santa Cruz Biotechnology). Vinculin (Goat α-vinculin primary (1:5,000) followed by a Donkey α-Goat secondary (1:5,000) (both Santa Cruz Biotechnology) was used as a loading control as previously shown (Hazell et al. 2019). Protein bands were visualised with Luminata Forte Western HRP substrate (Millipore Corporation, Billerica, MA, USA) using a G BOX (Syngene, Cambridge, UK) and densitometry was determined using Image J (developed at the National Institutes of Health and freely available at http://rsb.info.nih.gov).

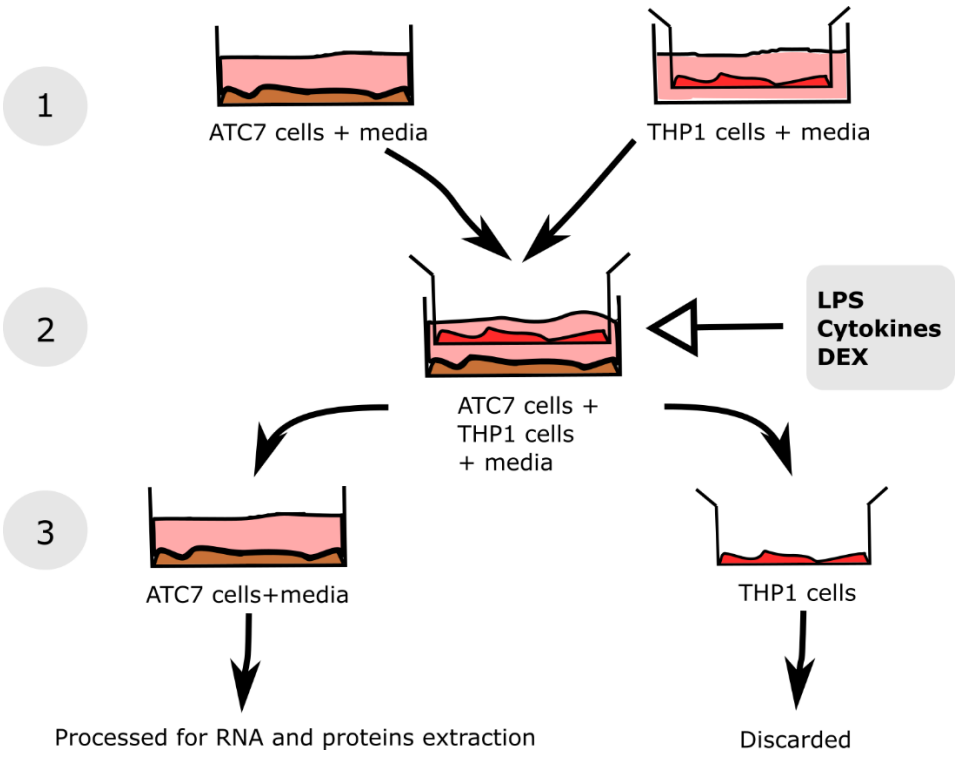

Figure 12 Diagram of the experiment plan. The ATC7 cells and the THP1 cells are first cultured separately (1) then are co-incubated using a transwell system and undergo various treatments with LPS, DEX or cytokines (2). At the end of treatment, the well containing the THP1 cells is removed, and the ATC7 cells only are collected for further processing for mRNA and protein measurement by RT-qPCR and western immunoblotting, respectively (3).

9.3.4 Statistics

Graph Pad Prism version 7.00 (Graph Pad Software, La Jolla, CA, USA) and SPSS version 24 (IBM Corp., Armonk, NY, USA) was used for data graphing and statistical analysis, respectively. All data are expressed as mean ± SEM. For all experiments, one-way, two-way or three-way analysis of variance (ANOVA) was used. When a significant effect of main factors or interactions were found, a Tuckey (One-way and two-way ANOVA) or Fisher's LSD (three-way ANOVA) multiple comparison tests were used. Significance was set at $P<0.05$.

9.4 Results

9.4.1 Effects of LPS stimulation of isolated ATC cells

The rationale of these initial experiments was to understand if isolated adrenal cells express IL-6 mRNA after LPS stimulation. First, I proved that the TLR4 mRNA is expressed in the adrenocortical cell lines. Analysis of the adrenal TLR4 mRNA revealed no effect of LPS stimulation dose and time course of treatment (1-hour and 6-hour treatments). I then moved to (1) to asses which cell lines are more responsive to LPS stimulation (ATC1 or ATC7), (2) the optimal dose of LPS, (3) the optimal time of LPS stimulation. I found that stimulation with LPS 10µg/mL for 24 hours achieved the most pronounced IL-6 mRNA response; however, this did not reach statistical significance (Figure 13) Furthermore, the ATC7 cells displayed a more pronounced response than the ATC1 cells.

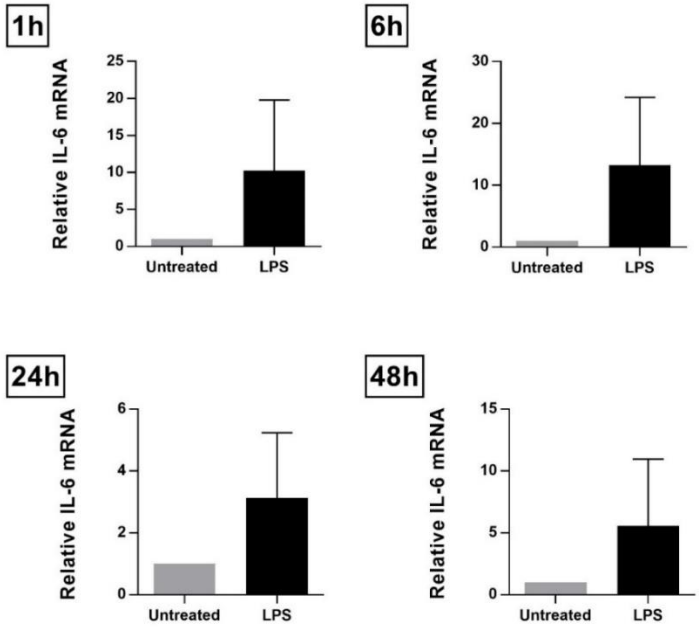

Figure 13 ACT7 cells were treated with LPS (10 µg/mL) for 1, 6, 24 and 48 hours. Relative levels of Il-6mRNA were measured by RTqPCR, and IL-6 mRNA expression was normalised to GAPDH. Data are mean ± SEM of three separate experiments and are expressed as fold induction of untreated ATC7 cells. Data were analysed using unpaired samples Student's t-test.

Because the IL-6 mRNA response remained modest, I explored (1) the effect of age of adrenocortical cells passage (“young” vs “old” cells) on the IL-6 mRNA expression, (2) the effect of changing the LPS batch used for stimulation, (3) the effect of ACTH co-incubation on IL-6 mRNA expression, however, I found no significant effect on any of the hypothesis tested. In the macrophage murine cell line, RAW 264.7, IFN-γ was shown to increase the affinity and number of GR receptor (Salkowski and Vogel 1992; Smyth et al. 2004). To my knowledge, the effect of IFN-γ on the expression of the pro-inflammatory cytokines in adrenal cells has not been studied; therefore, I hypothesised that co-treating the cells with IFN-γ could increase the responsiveness of the ATC7 cells, but again I found no significant effect (Figure 14).

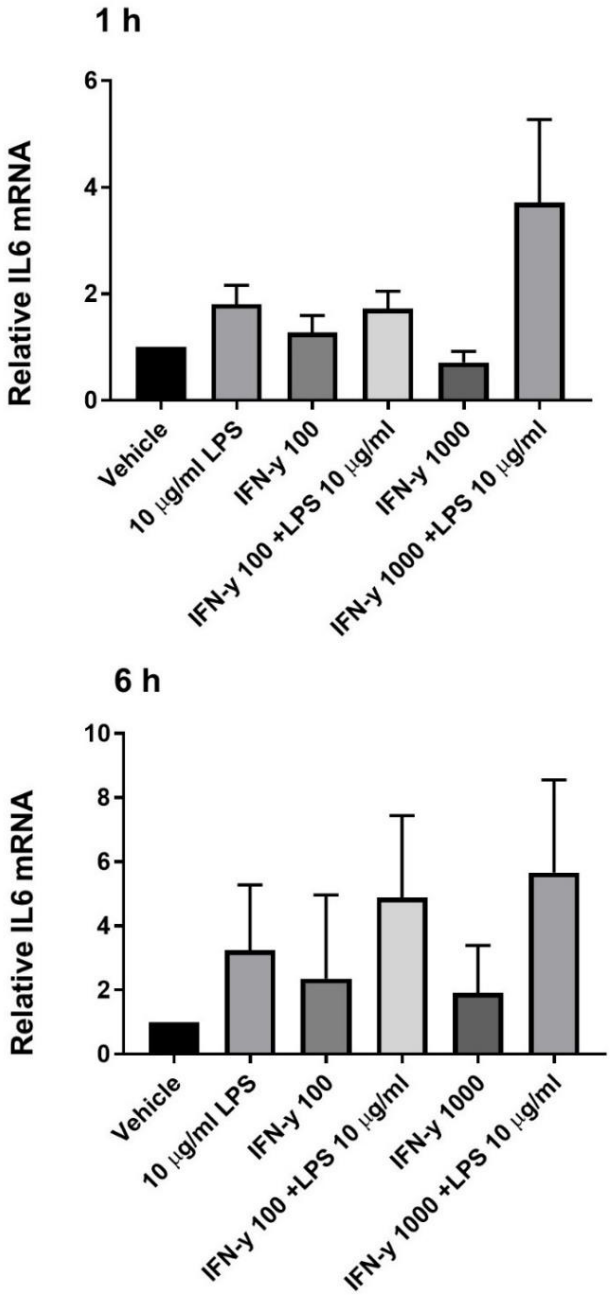

Figure 14 Effect of IFN-γ and LPS on IL-6 mRNA in ATC7 cells. ACT7 cells were treated with LPS (10 μg/mL) and/or IFN-γ (100u or 1000u) for 1 and 6 hours. Relative levels of IL-6 mRNA were measured by RTqPCR, and the IL-6 expression was normalised to GAPDH. Data are mean ± SEM of three separate experiments and are expressed as fold induction of untreated ATC7 cells. Data were analysed using unpaired samples Student's t-test.

9.4.2 LPS stimulation of ATC7 cells co-cultured with THP1 cells induces the expression of adrenal IL-6 mRNA

My preliminary experiments demonstrated no significant changes in the expression of IL-6 mRNA in response to LPS stimulation, either alone or co-incubated with IFN-γ. Because resident macrophages are found in basal unstimulated conditions in the adrenal cortex *in vivo*, I hypothesised that ATC7 cells would require the presence of activated immune cells for LPS to be able to affect the expression of pro-inflammatory cytokines and steroidogenic genes. Therefore, in this experiment, I tested the effect of co-culturing ATC7 cells with of THP1 derived macrophages (referred to as THP1) cells at various ratios, as well as the effect of treatment with various doses of LPS for 24 hours (Figure 15). Two-way ANOVA showed a significant effect of LPS ($P<0.0003$), but no effect of THP1 co-culture, nor interactions, was observed on Il-6 mRNA (Figure 15 A). Although higher levels of IL-6 mRNA could be observed in co-cultured ATC7 cells co-cultured with THP1 cells treated with LPS, post hoc test did not detect any specific difference between experimental groups. Next, I evaluated the dose-response effect of 24-hour LPS stimulation on ATC7 cells co-cultured with THP1 cells co-cultured at 1:2 ATC7 cells co-cultured with THP1 cells ratio. One-way ANOVA revealed a significant effect of LPS on IL-6 mRNA expression ($p=0.0032$; Figure 15 B), with a significant increase observed in cells treated with LPS at the dose of 1.25 µg /ml and 5 µg /ml concentration ($P=0.0453$ and $P= 0.0024$, respectively).

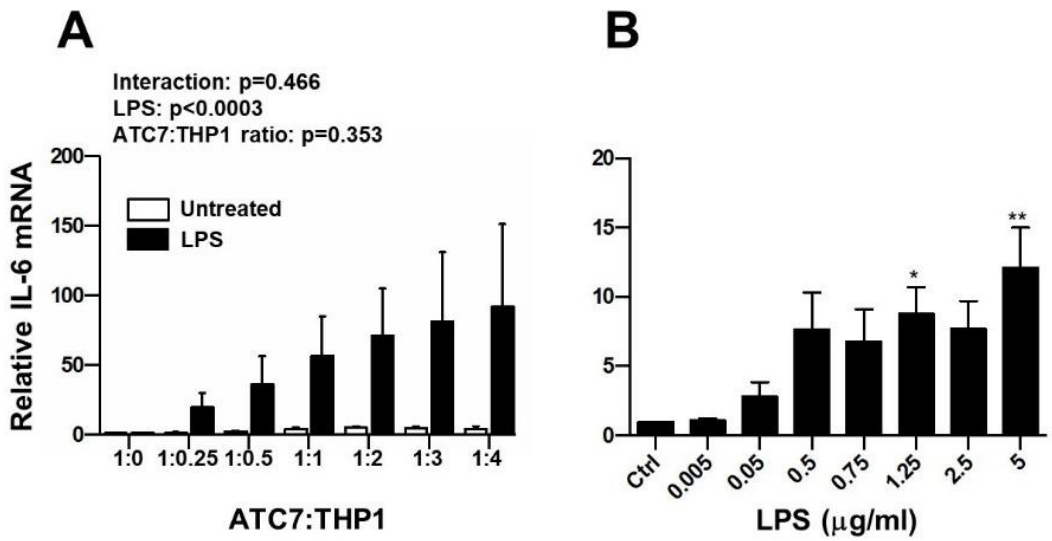

*Figure 15 Effect of LPS on adrenocortical ATC7 cells IL-6 mRNA expression. ATC7 cells were co-cultured with THP1 cells and/or treated with LPS (5μg/mL) for 24-h. Relative levels of Il-6 and steroidogenic gene mRNA were measured by RTqPCR and the expression of each target gene was normalised to GAPDH. (A) Effect of increasing ATC7:THP1 cells ratio and LPS treatment on IL6-mRNA expression in ATC7 cells. Data are the mean ± SEM of four separate experiments and are expressed as fold induction of untreated ATC7 (1: 0) cells. Data were analysed by two-way ANOVA followed by Tukey's multiple comparison test. (B) Effect of increasing doses of LPS in ATC7-THP1ATC7 cells co-cultured with thp1 cells (1:2 ratio) on IL6 mRNA expression in ATC7 cells. Data are the mean ± SEM of four separate experiments and are expressed as fold induction of untreated ATC7-thp1ATCatc7 cells co-cultured with thp1 cells (Ctrl). Data were analysed by one-way ANOVA followed by Tukey's multiple comparison test. *P<0.05; **P<0.01 vs Ctrl.*

9.4.3 Effect of increasing ratio of THP1 co-culture and LPS stimulation on steroidogenic gene expression in ATC7 cells.

Significant effects of ACT7-THP1 cells co-culture and LPS treatment were also found on the expression of key steroidogenic genes (Figure 16). Specifically, there was an overall effect of LPS on StAR mRNA (P=0.021; Figure 16 A) and an overall effect of THP1 co-culture on MC2R mRNA levels (P=0.001; Figure 16 B). As observed for Il-6 mRNA, *post hoc* analysis did not reveal any significant differences between groups, however, StAR mRNA levels appeared reduced in LPS-treated ATC7 cells co-cultured with THP1 cells, compared to

untreated ATC7 cells co-cultured with THP1 cells, and MC2R mRNA levels were elevated in ACT7-THP1 cells with low THP1 ratio (0.25 and 0.5), compared to single ATC7 cells (Figure 16 B). No effects of co-culture, nor LPS, were found on MRAP mRNA (Figure 16 C) or SF-1 mRNA (Figure 16 D). However, a significant effect of THP1 ($P<0.0001$), as well as a significant effect of THP1xLPS interaction ($P=0.048$), was found on DAX-1 mRNA (Figure 16 E). *Post hoc* testing revealed a significant decrease of DAX-1 mRNA in ATC7 cells co-cultured with THP1 cells treated with either LPS or vehicle. Interestingly, in ATC7 only cells, there was a trend of increase in the expression of DAX-1 mRNA in response to LPS stimulation ($p=0.072$) compared to ATC7 cells treated with vehicle.

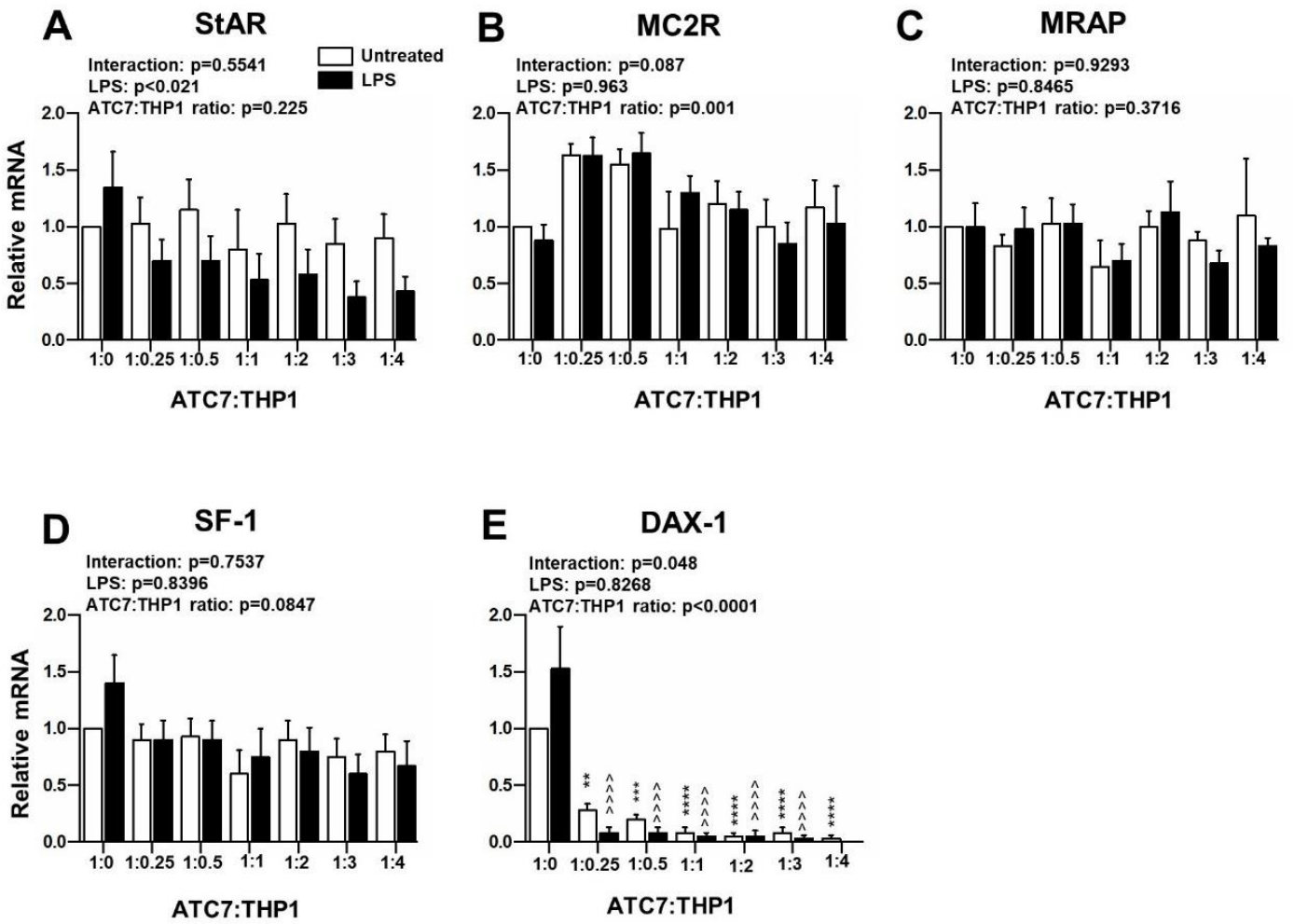

*Figure 16 Effect of increasing THP1 cells ratio and LPS stimulation on steroidogenic gene expression in ATC7 cells. ACT7 cells were co-cultured with THP1 and/or treated with LPS (5µg/mL) for 24-h. Relative levels of Il-6 and steroidogenic gene mRNA were measured by RTqPCR and the expression of each target gene was normalised to GAPDH. Data are mean ± SEM of four separate experiments and are expressed as fold induction of untreated ATC7 cells (1: 0); data were analysed by two-way ANOVA followed by Tukey's multiple comparison test. **P<0.01; ****P<0.0001 vs untreated ATC7 (1:0) cells; ^^^^ P<0.0001 vs LPS-treated ATC7 (1:0) cells.*

9.4.4 Dose-dependent effects of LPS on the expression of steroidogenic genes in ATC7 cells co-cultured with THP1 cells.

In this experiment, I evaluated the dose-response effect of 24-hour LPS stimulation on the expression of steroidogenic genes in ATC7 cells co-cultured with THP1 cells at a 1:2 ATC7 to THP1 cells ratio (Figure 17). Two-Way ANOVA showed a significant effect of LPS on StAR mRNA ($P<0.0001$; Figure 17 A) and DAX-1 mRNA ($P<0.0001$; Figure 13 E). Compared to controls, StAR mRNA expression was significantly decreased in cells treated with LPS at doses between 0.05 and 5µg/mL, ($p<0.0001$; Figure 17 B), whereas a significant decrease in DAX-1 was observed in cells treated with LPS at doses between 0.5 and 5µg/mL (Figure 13E). Consistent with the previous experiment, there was no effect of LPS on MC2R, MRAP and SF-1 mRNA (Figure 17 B-D). In accordance with the mRNA data, analysis of StAR protein showed a significant effect of LPS ($P<0.0001$; Figure 17 F), with a significant decrease in cells treated with LPS doses between 0.75 and 5µg/mL.

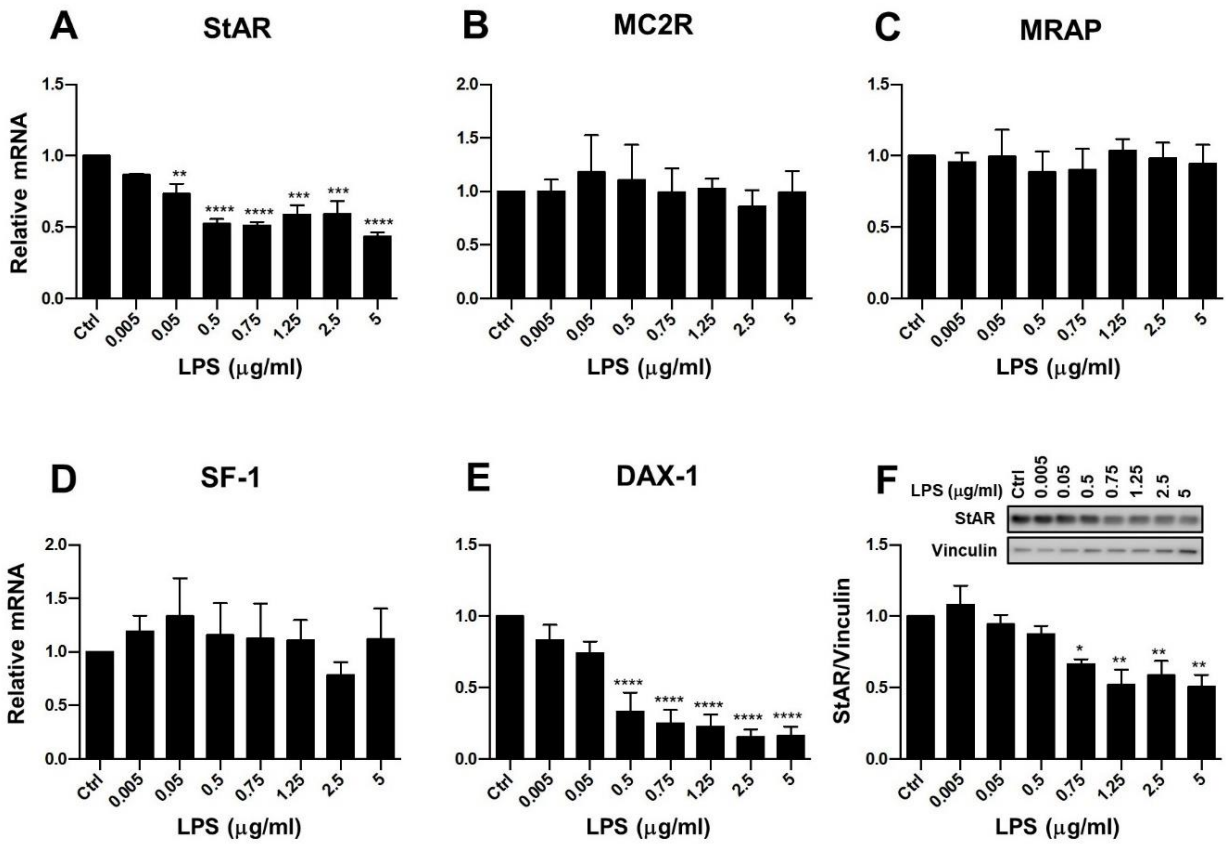

*Figure 17 Effect of LPS on ATC7 cells steroidogenic pathway. ATC7 cells were co-cultured with THP1 at 1:2 ratio and treated with LPS (5μg/mL) for 24-h. (A-E) Effect of LPS on steroidogenic genes mRNA expression. Relative levels of Il-6 and steroidogenic genes mRNA were measured by RTqPCR, and the expression of each target gene was normalised to GAPDH. (F) Effect of LPS on StAR protein expression in ATC7-thp1ATCatc7 cells co-cultured with thp1 cells. Relative levels of StAR protein were measured by western immunoblotting, and data were normalised to vinculin. Data are mean ±SEM of four separate experiments and are expressed as fold induction of untreated ATC7-thp1ATCatc7 cells co-cultured with thp1 cells (Ctrl); data were analysed by one-way ANOVA followed by Tukey's multiple comparison test. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 vs Ctrl.*

9.4.5 Effects of dexamethasone and LPS co-treatment on Il-6 and steroidogenic gene mRNA levels in ATC7 cells co-cultured with THP1 cells.

In the following sets of experiments, I tested the hypothesis that the effects of LPS on IL-6 mRNA and steroidogenic gene expression can be modulated by treatment with the synthetic glucocorticoid dexamethasone (DEX). Firstly, I investigated the effect of 24-hour co-treatment with DEX and LPS on the expression of IL-6 mRNA and steroidogenic genes mRNA in ATC7 cells co-cultured with THP1 cells (Figure 18). I found a significant overall effect of LPS ($P<0.0001$) on IL-6 mRNA, but no significant effect of DEX nor interactions (Figure 5 A). *Post hoc* testing revealed a significant increase in IL-6 mRNA in cells treated with 5 µg/mL LPS ($P=0.0009$) compared to control ATC7 cells co-cultured with THP1 cells, and this effect was prevented by both 1nM and 10nM DEX, but not by 100nM DEX ($P=0.0037$) compared to control ATC7 cells co-cultured with THP1 cells ((Figure 18 A). I did not observe any effect of the lower dose of LPS (0.05µg/mL LPS), nor an effect of DEX alone or in the presence of 5µg/mL LPS.

Analysis of the effects of DEX and LPS on steroidogenic gene expression revealed a significant effect of DEX ($P<0.0001$) and a significant DEX x LPS interaction ($P=0.0009$) on StAR mRNA (Figure 18 B). StAR mRNA levels were decreased in cells treated with 5µg/mL LPS ($P=0.0037$), compared to control ATC7 cells co-cultured with THP1 cells, and these effects were prevented by 1 nM DEX, but not by 10 and 100nM DEX. I also observed a significant decrease in StAR mRNA in cells treated with both 1nM DEX and 0.05µg/mL LPS ($P=0.0116$) compared to control ATC7 cells co-cultured with THP1 cells, suggesting a synergistic effect of DEX and LPS at low doses. There was also a significant effect of DEX ($P=0.0048$) and DEX x LPS interaction ($P=0.0051$) on DAX-1 mRNA (Figure 18 F). However,

post hoc analysis did not reveal any significant effect of LPS or DEX alone, but a trend of decrease in DAX-1 mRNA levels was found in cells treated with 1nM DEX and 0.05µg/mL LPS and in cells treated with 100nM DEX and 5µg/mL LPS (P=0.0887 and P=0.0800, respectively, compared to control ATC7 cells co-cultured with THP1 cells). Co-treatment with DEX and LPS did not affect MC2R, MRAP or SF-1 mRNA levels (Figure 18 C-E).

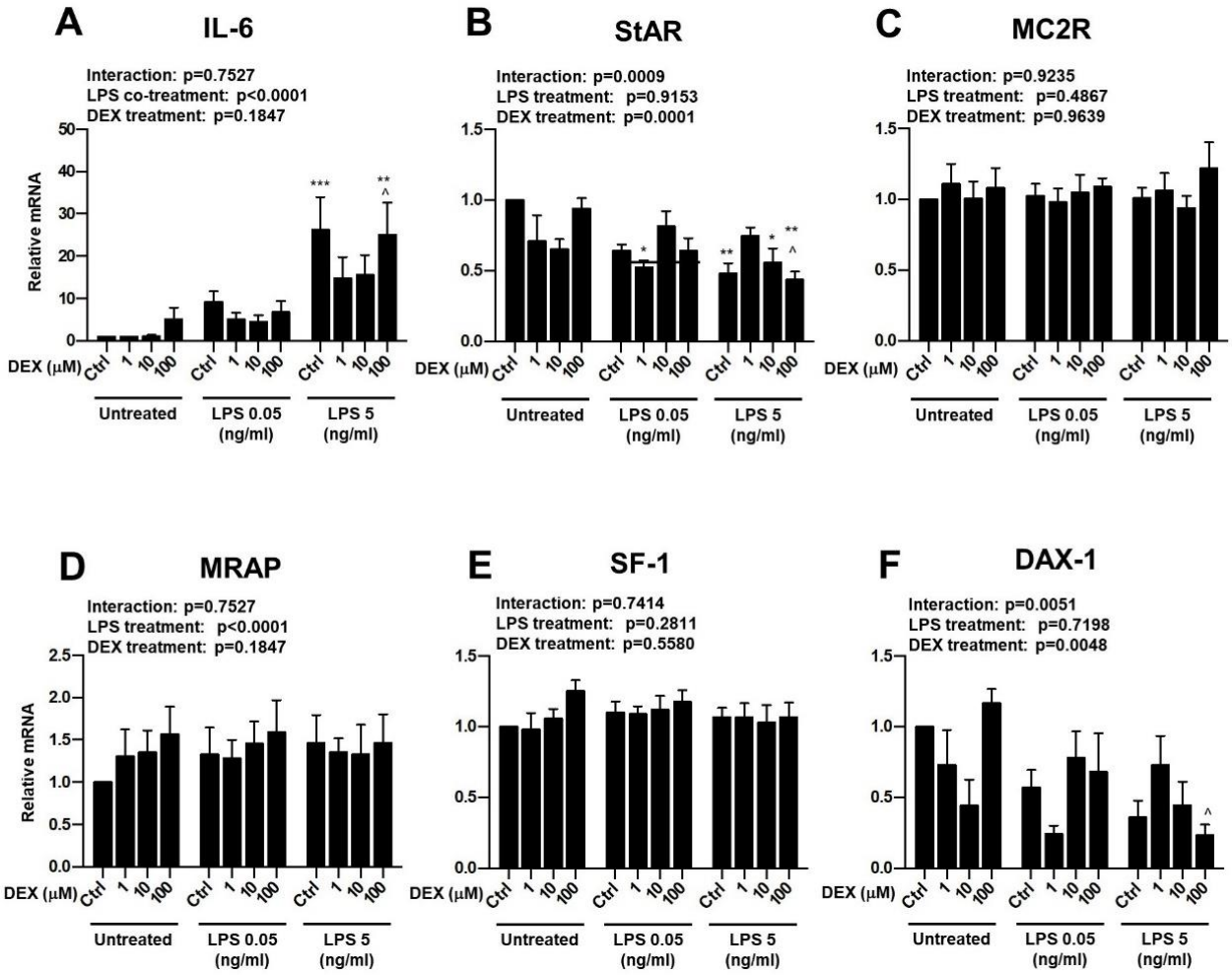

*Figure 18 Effect of dexamethasone and LPS on ATC7 cells IL-6 and steroidogenic genes mRNA expression. ATC7 cells co-cultured with thp1 cells were treated with dexamethasone (DEX, 1, 10 and 100 nM) and/or LPS (0.05 and 5 µg/mL) for 24-h. Relative levels of Il-6 and steroidogenic genes mRNA were measured by RTqPCR, and the expression of each target gene was normalised to GAPDH. Data are the mean ±SEM of six separate experiments and are expressed as fold induction of untreated Ctrl cells; data were analysed by two-way ANOVA followed by Tukey's multiple comparison test. *P< 0.05; **P< 0.01 vs untreated Ctrl; ^P<0.05; ^^P<0.01 vs untreated cells from the same DEX treatment group.*

9.4.6 Effects of dexamethasone pre-treatment on LPS-induced changes in IL-6 and steroidogenic gene mRNA levels in ATC7 cells co-cultured with THP1 cells.

My previous experiment has shown that, in ATC7 cells co-cultured with THP1 cells, treatment treated with DEX prevents some of the effects of LPS on IL-6, StAR and DAX-1 mRNA, but only at the lower doses of 1nM and 10nM. In this experiment, I have tested if glucocorticoids exert a bimodal effect (See chapter 9.13) on the expression of adrenal IL-6 mRNA and the changes in the steroidogenic gene pathway. In this experiment, I aimed to test whether pre-treatment with 100nM DEX was able to prevent LPS-induced effects on gene transcription in ATC7 cells co-cultured with THP1 cells. Twenty-four hours treatment with DEX was followed by 24-hours treatment with LPS (at the dose of 0.05 of 5 µg/mL) alone or in combination with DEX (Figure 19). Three-way ANOVA revealed a significant effect of LPS on IL-6 mRNA ($P<0.00001$) but no effect of DEX pre-treatment, DEX co-treatment, nor interactions (Figure 19 A). IL-6 mRNA levels were significantly higher in cells treated with 5nM LPS, and this effect was prevented in cells pre-treated with DEX, but not in cells both pre- and co-treated with DEX. Three-way ANOVA also revealed a significant effect of LPS on StAR ($P<0.0001$; Figure 19 B) and DAX-1 mRNA ($P<0.0001$; Figure 19 F), but no effect of DEX pre-treatment, DEX co-treatment, nor interactions on either gene. LPS treatment decreased StAR mRNA levels and neither pre- nor co-treatment with DEX prevented these effects. Similarly, LPS treatment decreased DAX-1 mRNA levels, but this effect was prevented in cells treated with 0.05µg/mL LPS pre- and co-treated with DEX. I also observed an overall effect of LPS on MC2R mRNA, ($P=0.0180$; Figure 19 C); however, a significant decrease in MC2R mRNA was only observed in cells treated with 5µg/mL LPS and pre-treated with DEX. Finally, a significant effect of DEX co-treatment was observed on MRAP mRNA (Figure 19

D); however, post hoc analysis did not reveal any significant difference between specific treatment groups.

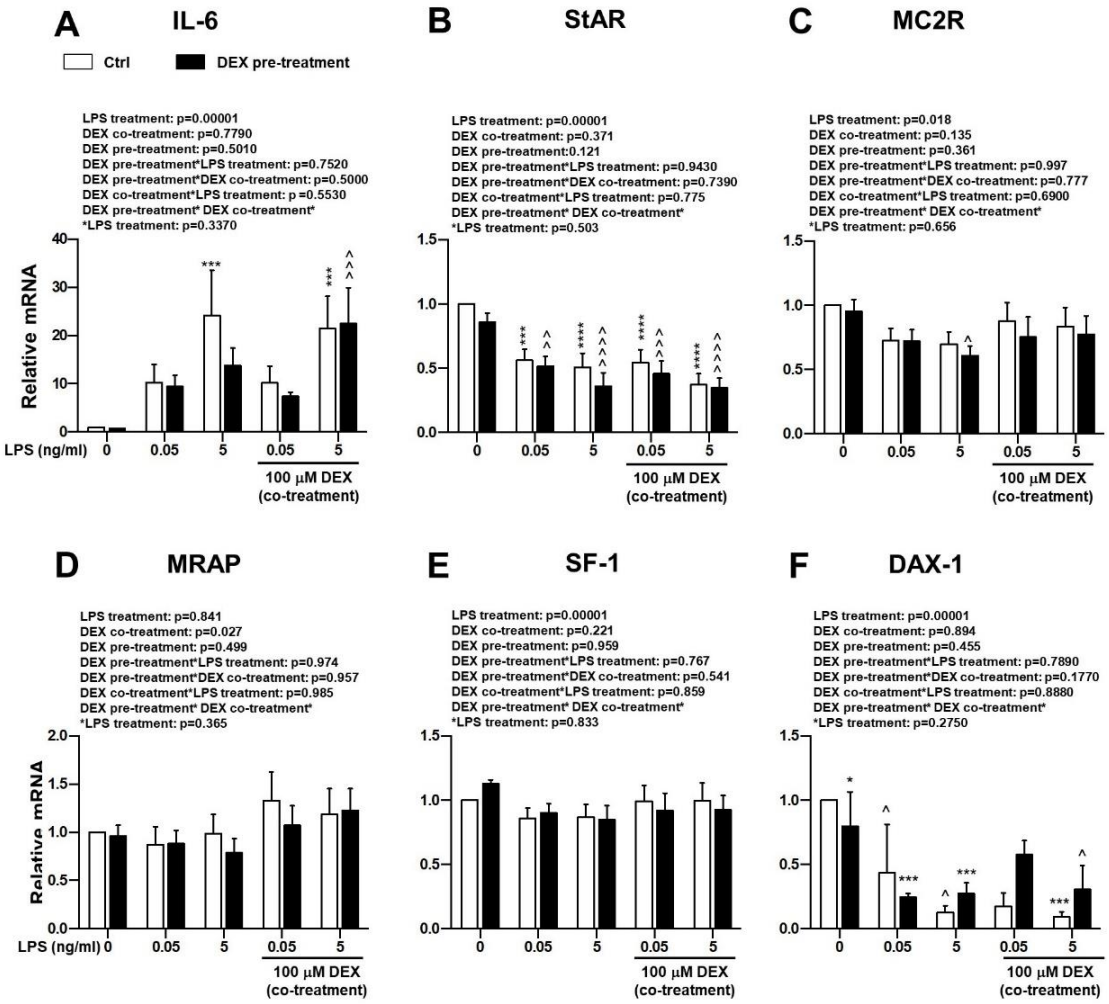

*Figure 19 Effect dexamethasone pre-treatment on dexamethasone and LPS effects on ATC7 IL6 and steroidogenic genes mRNA expression. ATC7-THP1ATC7 cells co-cultured with THP1 cells were pre-treated with dexamethasone (100 nM) for 24-h, and then co-treated with LPS (0.05 or 5 µg/mL) and/or dexamethasone (100 nM) for 24-h. Relative levels of Il-6 and steroidogenic genes mRNA were measured by RTqPCR, and the expression of each target gene was normalised to GAPDH. Data are mean ±SEM of six separate experiments and are expressed as fold induction of untreated Ctrl; data were analysed by three-way ANOVA followed by Fisher's LSD post hoc test. ***P< 0.001; ****P< 0.0001 vs LPS-untreated Ctrl; ^P<0.05; ^^P<0.01 ^^^P<0.001; ^^^^P<0.0001 vs Ctrl cells from the same LPS±DEX treatment. The closed bars denote DEX pre-treated cells.*

9.4.7 Effect of LPS on ACTH- induced IL-6 mRNA and steroidogenic gene expression in ATC7 and ATC7 cells co-cultured with THP1 cells

Studies in humans and rodents have shown that LPS-induced glucocorticoid secretion can occur through its effects on the HPA axis (George P. Chrousos 1995). In addition to regulating the secretion of CRH in the hypothalamus, and of ACTH in the pituitary, LPS administration directly activates the adrenal gland steroidogenic pathway and can potentiate the effects of ACTH on glucocorticoid synthesis (Kanczkowski, Sue, and Bornstein 2016). Therefore, I decided to investigate the effects of LPS treatment on Il-6 and steroidogenic genes mRNA in both ATC7 alone and ATC7 cells co-cultured with THP1 cells (Figure 20). In these experiments set, ATC7 and ATC7 cells co-cultured with THP1 cells were treated with LPS 5µg/mL for 24 hours and then treated with ACTH 10nM for up to 2h.

Three-way ANOVA analysis of Il-6 mRNA data showed a significant effect of ACTH ($P<0.0001$), LPS ($P<0.0001$), THP1 ($P=0.02$) as well as ACTH x THP1 ($P=0.007$), LPS x THP1 ($P<0.0001$) and ACTH x LPS ($P=0.011$) interactions (Figure 20 A). Interestingly, I found that ACTH alone increased IL-6 mRNA levels in ATC7 cells, and this effect was potentiated by pretreatment with LPS. Interestingly, ACTH alone did not increase Il-6 mRNA in ATC7 cells co-cultured with THP1 cells, whereas a significant increase was observed when ATC7 cells co-cultured with THP1 cells were treated with LPS.

Analysis of StAR mRNA showed a significant effect of ACTH ($P<0.0001$), LPS ($P=0.001$) and THP1 ($P<0.0001$) as well as a significant ACTH x THP1 interaction ($P=0.005$) (Figure 20 B). As expected, StAR mRNA levels were increased in ATC7 cells treated with ACTH, and LPS did not affect such effect. However, the increase in StAR mRNA induced by ACTH was reduced in ATC7 cells co-cultured with THP1 cells, an effect that was further

potentiated by LPS. A significant effect of ACTH (P<0.0001) and THP1 (P=0.021), as well as THP1 x LPS interaction (P=0.049), was also observed on MC2R mRNA levels (Figure 20 C). However, while there were no significant changes in ATC7 cells treated with ACTH, even following pre-treatment with LPS, MC2R mRNA levels were higher in ATC7 cells co-cultured with THP1 cells treated with ACTH only, when compared to ATC7 cells. DAX-1 mRNA levels were also affected by both ACTH (P=0.031) and THP1 (P<0.0001), with a significant effect of ACTH x THP1 interaction (P=0.024), whereas only a trend of the effect of LPS was observed (P=0.082) (Figure 20 F). DAX-1 mRNA levels were decreased in ATC7 cells treated with ACTH±LPS at 2h, compared to time 0, whereas a significant decrease was observed in ACT7-THP1 cells prior to ACTH treatment, and no further decrease was observed after ACTH treatment, nor LPS treatment had any further effect. Only a trend of effect of ACTH was observed on MRAP (figure 16D), while a significant effect of THP1 (P<0.0001), and a trend of effect of LPS (P=0.09), was found on SF-1 mRNA levels, with a significant overall decrease in ATC7 cells co-cultured with THP1 cells treated with LPS (Figure 20 E).

To evaluate whether the decrease in ACTH-induced StAR mRNA in ATC7 cells co-cultured with THP1 cells was associated with a decreased activation of CREB, I measured the levels of pCREB using Western immunoblot (Figure 20 G). Although there was no significant effect of ACTH, LPS or THP1, a significant ACTH x LPS interaction was detected (P=0.024). Post hoc analysis revealed that while ACTH increased pCREB levels in ATC-7 cells pretreated with vehicle (P=0.02), only a trend of effect was found in ATC-7 cells pre-treated with LPS (P=0.066), and no significant effect of ACTH was found in ATC7 cells co-cultured with THP1 cells.

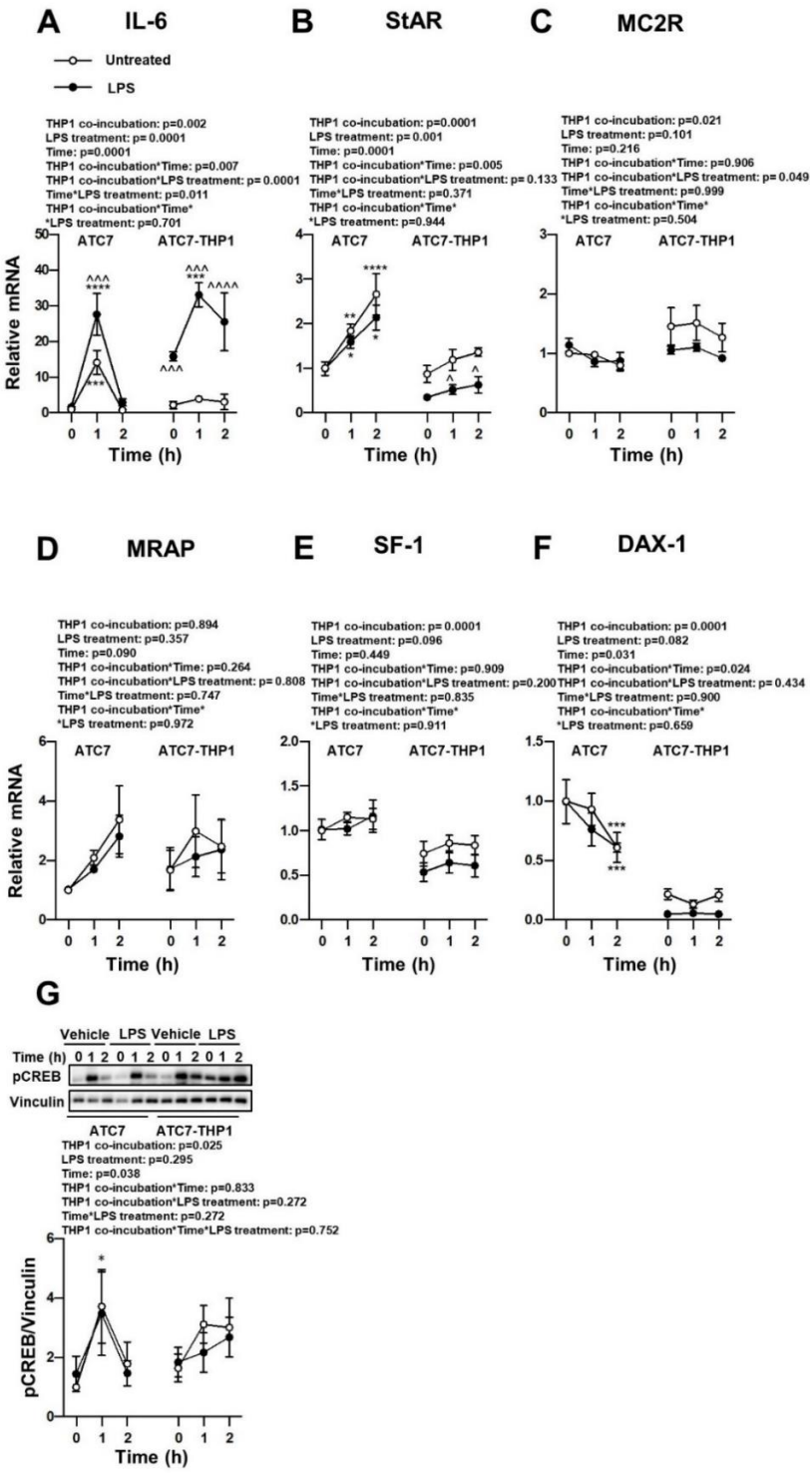

*Figure 20 Effect of LPS on ACTH-induced IL6 mRNA and steroidogenic pathway activity. ATC7 and ATC7-THP1ATC7 cells co-cultured with THP1 cells were incubated with LPS (5 µg/mL) and then treated with ACTH for up to 2 h. (A-F) Il-6 and steroidogenic genes mRNA levels were measured by RTqPCR and data from each target gene were normalised to GAPDH. (G) Relative levels of phosphorylated CREB (pCREB) was measured by western immunoblotting, and data were normalised to vinculin. Data are mean ±SEM of four separate experiments and are expressed as fold induction of untreated ATC7 cells; data were analysed by three-way ANOVA followed by Fisher's LSD post hoc test. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 vs same treatment ATC7 or ATC7 cells co-cultured with THP1 cells at time 0; ^^P<0.01; ^^^P<0.01; ^^^^P<0.01 vs untreated ATC7 or ATC7 cells co-cultured with THP1cells at the same time*

9.5 Discussion

Recent research has demonstrated the role of HPA axis-independent, intra-adrenal mechanisms in regulating glucocorticoid release during acute inflammatory stress (Boonen, Bornstein, and Van den Berghe 2015). Such mechanisms could likely complement or augment the well-known HPA axis activation during critical illness states. The adrenal tissue microenvironment contains a variety of cells, including neural cells, adipocytes, endothelial and immune cells, that could indeed regulate adrenal steroidogenesis (Boonen, Bornstein, and Van den Berghe 2015). The interaction of steroidogenic cells with immune cells is of particular importance because several studies have shown that the generalised inflammation that accompanies acute stress is associated with an infiltration of the adrenal cortex by immune cells (Jennewein et al. 2016; Kanczkowski, Chatzigeorgiou, et al. 2013). This immune-steroidogenic crosstalk could occur either following activation of resident macrophages or/and by the intervention of circulating immune cells that are recruited in the adrenal cortex. One study suggested that systemic immune cells, rather than the adrenal cells, are the primary regulator of the TLR-mediated adrenal activation (Kanczkowski, Alexaki, et al. 2013). It is undoubtedly the case that the adrenals glands, like the thyroid gland, have the highest blood supply *per* gram of tissue in the body, and the adrenal tissue will likely be flooded by immune-effector cells during acute inflammatory stress.

The current study reports the characterisation of a novel co-culture model to investigate these interactions. The use of the adrenocortical tumour ATC7 cell line with complete *zona fasciculata* cell phenotype enabled us to assess the effect of an inflammatory stimulus on the expression of the pro-inflammatory cytokine IL-6 mRNA and the expression of key steroidogenic genes. Rat and human adrenal cells do express a variety of pro-inflammatory cytokines in response to immune activation including TNFα, IL-1, IL6, IL18, TGFβ (Judd

1997; Bornstein, Rutkowski, and Vrezas 2004). I have chosen to measure the expression of IL-6 because it can be induced by inflammation directly as well as in response to IL-1β. Furthermore, several studies have shown that IL-6 can affect adrenal steroidogenesis either directly or via activation of the CRH-ACTH axis (G P Chrousos, Branch, and Development 2015; Bethin, Vogt, and Muglia 2000; Bornstein, Rutkowski, and Vrezas 2004). In humans, the presence of IL-6, IL-6 receptor and IL-6 mRNA in the adrenal cortex suggests that IL6 could play a paracrine or autocrine role in the immune adrenal cross-talk (Gonzalez-hernandez and Scherbaum 2016; Päth et al. 1997). I decided to use the THP1 cell line because this is a broadly used model to study the monocyte/macrophage functions (Tsuchiya et al. 1980). THP1 cells have been used before in other co-culture models including vascular smooth muscle cells (Zhang et al. 2008; Li et al. 2006) , adipocytes (Spencer et al. 2010) , T-lymphocytes (Azenabor et al. 2011), platelets (Aslam et al. 2007) and intestinal cells (Watanabe et al. 2004). Furthermore, THP1 cells, and particularly the matured macrophages, are known to secrete several pro-inflammatory cytokines as a result of LPS stimulation including TNFα, IL-1β, IL-6, IL-8 and IL-10 (Schildberger et al. 2013; Wehrhahn et al. 2010; Palacio, Markert, and Martínez 2011).

In the present study, I show a significant increase in IL-6 mRNA expression in ATC7 cells in response to LPS only when these cells are co-cultured with the THP1 cells, suggesting that the expression of adrenal pro-inflammatory cytokines in response to inflammatory stress is dependent on the presence of immune cells. Because LPS had no effect on ATC7 alone, I hypothesise that, in our co-culture experimental model, LPS induces the secretion of a cytokine mix by THP1 macrophages which then acts on the adrenal cells resulting in the expression of IL-6 mRNA. I have also found that the effects of LPS on Il-6 mRNA is dependent on the ATC7 to THP-1 cells ratio. This suggests that *in vivo*, the increased expression of adrenal pro-

inflammatory cytokines during acute stress could occur by increased recruitment of immune cells within the adrenal cortex.

I have also assessed the effect of glucocorticoids on immune-adrenal interactions. This approach is novel since, to my knowledge, the effects of glucocorticoids on the HPA axis responses to inflammation has only been investigated at a system level, and not directly in the adrenal gland cells. I found a significant effect of a high dose of LPS on the increase of IL-6 mRNA expression. This increase was suppressed by low and medium DEX doses. A similar dose-dependent suppression was noted in the StAR mRNA expression as a result of LPS stimulation. Furthermore, I found a significant effect of DEX on DAX-mRNA response to LPS stimulation, whereas we noted a trend in a decrease of DAX-1 mRNA expression dependent on LPS and DEX dose co-stimulation. Gummow et al. investigated the direct effect of dexamethasone on the steroidogenic gene expression in primary adrenocortical cells, and they found an increase in DAX-1 mRNA expression and a decrease in StAR mRNA expression that was mediated by glucocorticoid receptor activation (Gummow et al. 2006). In my experiments, I did not find any effect of dexamethasone co-incubation on steroidogenic gene expression in ATC7-THP1 cells in the absence of LPS co-stimulation. Nevertheless, my data further support a direct effect of glucocorticoids on the steroidogenic network activity. This suggests that during acute inflammatory stress, systemic administration of glucocorticoids can modulate the steroidogenesis directly, without the pituitary inputs (e.g. in HPA axis independent manner).

I also investigated the temporal relation between glucocorticoid and LPS stimulation in regulating the expression of IL-6 and steroidogenic genes. Despite traditional views according to which glucocorticoids are considered uniformly anti-inflammatory, research in the last decade has suggested that glucocorticoids can have a bimodal action: both pro-inflammatory and anti-inflammatory (Sorrells et al. 2009; Sapolsky, Romero, and Munck 2000). This bimodal effect seems to depend on the time of glucocorticoid administration in relation to the

inflammatory stress stimulus. A pro-inflammatory effect of glucocorticoids has been demonstrated in immune-competent cell lines (macrophages) (Smyth et al. 2004) and in the central nervous system (hippocampal microglia) (Frank et al. 2007). I investigated whether this effect occurs within the isolated adrenal cells depending on the time of glucocorticoid administration in relation to the inflammatory stress (LPS stimulation). I found that DEX pre-treatment prevented the LPS-induced IL-6 mRNA when compared to pre- and co-treated DEX cells. Therefore I can conclude that the so-called bimodal effect of steroids (anti- and pro-inflammatory) that was noted in immune and neural cell lines does not apply to adrenal cells, at least within the experimental conditions used in our studies. (Yeager, Guyre, and Munck 2004; Horowitz and Zunszain 2015).

Because ACTH plasma levels increase in response to inflammatory stress, I also investigated the effects of ACTH treatment on Il-6 and steroidogenic genes mRNA in both ATC7 alone and in ATC7 cells co-cultured with THP1 cells. To my surprise, I found that ACTH alone was able to induce IL-6 mRNA in ATC7 cells, and this effect was potentiated by pretreatment with LPS. Interestingly, the effect of ACTH on Il-6 mRNA was not observed in ATC7 cells co-cultured with THP1 cells in the absence of LPS, suggesting that anti-inflammatory cytokines secreted by THP1 cells in basal conditions may protect the adrenal cells from a non-inflammatory immune activation mediated by ACTH. A recent study has shown that ACTH treatment dynamically increases the expression of steroidogenic genes in ATC7 cells (Hazell et al. 2019). My present data confirmed these previous findings, but also show that the dynamic effect of ACTH is disrupted in ATC7 cells co-cultured with THP1 cells, with a smaller effect on StAR mRNA, which was further decreased by pre-treatment with LPS, and complete suppression of DAX-1 mRNA. These effects were associated with a decrease in pCREB levels, suggesting that the effects of co-culture with THP1 cells may occur at the levels of cAMP/PKA signalling. Interestingly, the effects of ACTH on other steroidogenic genes,

including MC2R, MRAP and SF-1 were not affected by co-culture with THP1 cells, nor by pre-treatment with LPS. The effect of ACTH on IL-6 mRNA and steroidogenic genes was significantly different in the presence of THP1 cells. IL-6 mRNA and phosphorylation of CREB appeared enhanced by ACTH in the presence to THP1 cells and LPS, while the suppression of STAR mRNA and DAX-1 mRNA was more pronounced in the LPS-treated cells, compared to vehicle-treated ATC-THP1 cells. A link between an increase in CREB phosphorylation and progesterone levels in response to IL-1b has been shown in *granulosa* cells (Dang et al. 2017). Therefore, it is tempting to speculate that the effects of immune stimulation in adrenocortical cells may occur by a similar mechanism. Overall my results suggest that immune-adrenal crosstalk may be integrated with the hormonal response of the HPA axis during acute stress.

9.6 Conclusions

I have shown that stimulation of isolated adrenal cells does not induce a significant expression of IL-6 mRNA. Therefore, I have developed a novel co-culture model suitable for assessing immune-adrenal interactions in the context of inflammatory stress. Using this system, I was able to demonstrate that the expression of pro-inflammatory adrenal cytokines after LPS stimulation is dependent on the ratio of adrenal and immune cells. I have also noted that the presence of THP1 cells can modulate the response of the steroidogenic gene network to LPS activation, and this is further modulated by ACTH stimulation and glucocorticoid incubation.

9.7 Suggestions for future work

The directions for future work should focus on a better understanding of the crosstalk between ATC-7 cells and THP-1 cells. This would involve measuring the adrenal specific

cytokines and macrophage-specific cytokine in the media followed by stimulation of isolated ATC7 cells with the cytokines of interest. Furthermore, it would be useful to assess if the rest of mRNA expression results are verified in terms of translation to protein levels. Finally, a further direction is to concentrate on the changes that occur within the THP1 cells during the interaction with ATC-7.

10 CHAPTER 3 THE UTILITY OF GLUCOCORTICOIDS IN PAEDIATRIC HEART SURGERY

"The absence of proof does not constitute the proof of absence."

Rudolf Virchow

Sections 10.1.1 to 10.1.5 of this chapter are largely taken from a published narrative review in Frontiers in Pediatrics (D. P. Fudulu, Schadenberg, et al. 2018). The author's contributions were the following: Daniel Fudulu - literature review, writing, design, supervision; Ben Gibbison, Thomas Upton, Serban Stoica, Massimo Caputo and Stafford Lightman - the writing of manuscript sections and revision; Gianni Angelini - writing, design and supervision.

10.1 Corticosteroids in Paediatric Heart Surgery: Myth or Reality

10.1.1 Background

The introduction of the CPB circuit in the mid-1950s made the surgical treatment of intracardiac lesions possible and led to rapid progress in the field of cardiac surgery (Cooley and Frazier 2000). However, the CPB circuit is also known to provoke a systemic inflammatory response syndrome (SIRS) due to the contact of the blood to the extracorporeal circuit, the ischemic reperfusion injury of the heart or endotoxemia due to increased gut permeability. This systemic activation is beneficial because it triggers immune priming of the body that could prevent infection and promote healing, but if excessive, can also prove detrimental and thus result in organ dysfunction and even death (Laffey, Boylan, and Cheng 2002). Therefore, since the introduction of the CPB, various strategies have been employed to modulate this systemic

inflammatory response to improve clinical outcomes. Such strategies include the use of glucocorticoids, aprotinin, antioxidants, miniaturised or heparin-coated circuits (D. Fudulu and Angelini 2016). Furthermore, in paediatric heart surgery, the modulation of SIRS is of greater importance because it is believed that the inflammatory response is augmented by the surface of the extracorporeal circuit relative to the reduced circulating blood volume, the more frequent use of the deep hypothermic circulatory arrest (DHCA) and the more pronounced haemodilution (Kouchoukos et al. 2012). Historically, the use of corticosteroids in cardiac surgery dates back to the 1960s (Replogle, Gazzaniga, and Gross 1966) and according to several current surveys of clinical practice, corticosteroids are still widely used in paediatric heart surgery with the use of CPB (P. A. Checchia et al. 2005; Allen et al. 2009). This contrasts with adult heart surgery where prophylactic corticosteroids are no longer widely used because of no clear evidence of a beneficial effect on clinical outcomes. The DECS trial (Dieleman et al. 2012) recruited 4494 adult patients undergoing surgery with the use of CPB and found no impact, of a single dexamethasone dose 1mg/kg given intraoperatively, on the composite endpoint (death, myocardial infarction, stroke, renal failure and respiratory failure at 30 days). However, in the secondary outcomes, dexamethasone was associated with reductions in postoperative infection, duration of mechanical ventilation and length on intensive care and hospital stays. The SIRS trial (Whitlock et al. 2015) remains the largest trial of corticosteroids versus placebo in adult cardiac surgery to date. In this trial, 7507 were randomly assigned to methylprednisolone 250 mg at anaesthetic induction and 250 mg at the initiation of CPB or placebo. Corticosteroids had no impact on the risk of death or significant morbidity including infection, length of the hospital, intensive care stay, respiratory or renal failure. The prophylactic use of corticosteroids in the paediatric cardiac surgery population continues to be a matter of debate likely due to the lack of such well-designed, large randomised controlled trials (RCT) that can detect a treatment effect in the context of the current low mortality and

morbidity. However, corticosteroids with (mineralocorticoid effects) are also given in paediatric heart surgery to protect against the so-called *relative adrenal insufficiency* that can accompany the acute stress of surgery (Boonen, Bornstein, and Van den Berghe 2015; Green and Koch 2012). Due to a lack of basic understanding of hypothalamic-pituitary axis physiology during and after paediatric heart surgery, the evidence is limited in this area (Graham and Bradley 2017). Finally, another potential use of corticosteroids in paediatric heart surgery is their neuroprotection effect during DHCA surgery. In this chapter, I will discuss the evidence and controversies around these three main indications of steroid use in paediatric heart surgery (Figure 21).

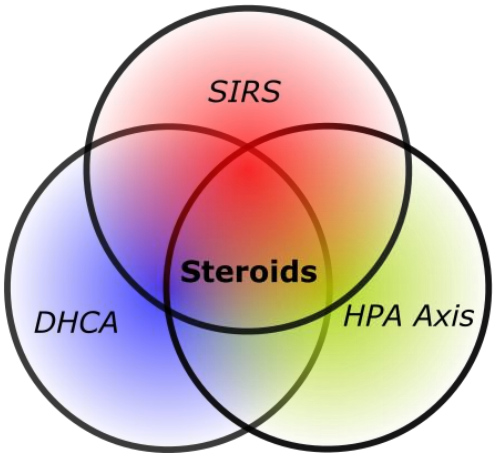

Figure 21 The three main glucocorticoid indications

10.1.2 Corticosteroids, inflammation and clinical outcomes

Many studies have tried to measure various markers of inflammation after glucocorticoid administration and attempted to correlate these changes to clinical outcomes. (Tennenberg et al. 1986; Bronicki et al. 2012; Varan et al. 2002; Schroeder 2003; Lindberg et al. 2003; Gessler et al. 2005; Grosek et al. 2007; Graham et al. 2011; Bocsi et al. 2011; Lerzo et al. 2011; Heying et al. 2012; Abbasi Tashnizi et al. 2013; Keski-Nisula et al. 2013; Byrnes et al. 2015) Firstly, it is well known that SIRS is a *multifaceted*, complex response that is

challenging to characterize and modulate. Therefore, measuring only a few cytokines might not be accurate enough given the array of pro-inflammatory and anti-inflammatory cytokines that are released during SIRS and interact in a complex manner (Jaffer, Wade, and Gourlay 2010). Secondly, while glucocorticoids can blunt inflammation; this does not necessarily translate into better short term clinical outcomes(Lindberg et al. 2003; Amanullah et al. 2016; Keski-Nisula et al. 2013). Moreover, a study by Gessler et al. found no impact of glucocorticoid administration on markers of inflammation (Gessler et al. 2005). Graham et al. in an RCT of 68 children undergoing surgery with the use of CPB found no effect single versus 2-dose corticosteroids on inflammation (Graham et al. 2014). Finally, there is some evidence that the *host inflammatory response* plays an important role, and it might be essential to approach SIRS in a personalised rather than standardised manner. Huber et al. (Huber et al. 2017) in a study of 37 children undergoing heart surgery with the use of CPB demonstrated that the neutrophil phenotype signature could predict end-organ dysfunction associated with SIRS.

There are several small-sized randomised controlled trials (RCTs) that focus on the effect of corticosteroids on clinical outcomes, too (Table 2). Most of these trials measure inflammation parameters, and the clinical data is measured either as a primary or secondary outcome. All have in common the small sample size and the variability in the type of steroid and the regimens used. The first randomised controlled trial of steroid (47 children) versus placebo (48 children) was published by Toledo-Pereyra et al. and dates to 1980. The authors found a benefit in survival in patients that had methylprednisolone 30 mg/kg: 1 hour preoperatively, five minutes pre-CPB and every 6 hours for 24 hours, however, there was no correlation with other biochemical markers (Toledo-Pereyra et al. 1980). Bronicki et al. in an RCT of 28 children, found dexamethasone given 1mg/kg, 1 hour before CPB, to result in the lower fluid requirement, lower alveolar-arterial gradients, lower fluid requirement and less mechanical ventilation (Bronicki et al. 2000). In an RCT of 30 children, Varan et al. compared

the dose-effect of 30 mg/kg of methylprednisolone intravenous infusion to 2 mg/kg methylprednisolone intravenous infusion pre-CPB. There was no difference in the clinical outcomes between the two groups (ventilation times, intensive care unit stay or oxygenation) (Varan et al. 2002). Later, Lindeberg and colleagues in an RCT of 40 children found no effect of dexamethasone 1mg/kg after anaesthesia induction on oxygenation, fluid balance, critical care stay or ventilation time (Lindberg et al. 2003). Schroeder et al. in an RCT of 29 children, compared the effect of 30 mg/kg of methylprednisolone double-regimen (pre-operatively and intraoperatively) to a single dose (30 mg/kg) intraoperatively. The double-dose steroid recipients had better oxygenation, lower body temperature and reduced fluid requirements. Checchia et al. reported in an RCT of 28 children no effect dexamethasone 1 mg/kg, given 1-hour pre-CPB on sternal dehiscence rates, reoperation for bleeding, or gastrointestinal bleeding (P. a Checchia et al. 2003). Ando et al. in small RCT of 20 patients found a benefit of hydrocortisone administration in terms of reduced body oedema, improved oxygenation and reduced duration of ventilation (Ando et al. 2005). Graham et al. randomised a total of 76 patients to either single (intraoperative) or double dose methylprednisolone (8-hour pre-operative and intraoperative). The two-dose steroid group had a higher serum creatinine and poorer postoperative diuresis (Graham et al. 2011). There was no difference in the incidence of low cardiac output syndrome, inotropic requirement, duration of mechanical ventilation, ICU or hospital stay. Heying et al. evaluated the effect of dexamethasone 1 mg/kg 4 hours pre-CPB in 20 neonates undergoing arterial switch and found no effect of corticosteroids in terms of postoperative cardiac parameters (heart rate, mean arterial pressure, central venous pressure), diuresis, oxygenation. However, there was a reduced dobutamine requirement, 4 hours post-CPB. (Heying et al. 2012). Keski-Nisula and colleagues, in an RCT of 40 neonates undergoing surgery with use of CPB, found no effect of 30 mg/kg of methylprednisolone given at induction of anaesthesia, on postoperative lactate, central venous saturation, inotropic score,

duration of ventilation or survival; however, the steroid arm had significantly increased blood glucose (Keski-Nisula et al. 2013). The same group investigated in a three-arm RCT of 45 children, the effect of 30 mg/kg methylprednisolone after induction versus 30 mg/kg methylprednisolone in prime versus placebo. There were no differences between the three groups, in terms of lactate, inotropic score, duration of ventilation and intensive care stay but the steroid arms had higher blood glucose compared to placebo (Keski-Nisula et al. 2015). Amanullah et al. in RCT of 152 children investigated the effect of dexamethasone 1mg/kg given at three time-points (induction, pre-CPB and 6 hours from last dose) versus placebo (Amanullah et al. 2016). There was no difference between the two arms in terms of ventilation times, urine output, mean systolic and diastolic pressure, central venous pressure, inotrope score at 6 hours and fluid requirement.

The largest RCT to date in neonates was recently published by Graham et al. (Graham et al. 2019) In this double-blinded RCT, the authors randomised 190 patients to either methylprednisolone (30 mg/kg) or a placebo after the induction of anaesthesia. The primary endpoint of the study was a morbidity-mortality composite that included any of the following events following surgery before discharge: death, mechanical circulatory support, cardiac arrest, hepatic injury, renal injury, or rising lactate level (>5 mmol/l). In the overall analysis, there was no statistically significant difference in reaching the primary endpoint between the groups (odds ratio [OR]: 0.63; 95% confidence interval [CI]: 0.31 to 1.3; p = 0.21). However, in the analysis by type of procedure (corrective versus palliative), methylprednisolone was associated with reductions in inotropic requirements and the incidence of the composite endpoint.

Table 2 Randomized controlled trials of steroid use in children.

First author	Year of publication	Sample size	Study design	Type of steroid	Dose	Route	Regimen	Effect steroids on Inflammation	Effect steroids on clinical outcomes	Benefit/No Benefit/Harm
Toledo-Pereyra et al.	1980	95 children	RCT steroid vs placebo	Methylprednisolone	30 mg/kg	IV	pre-op, pre-CPB, post CPB, every 6 hours for 24 hours	not assessed	Increased survival	Benefit
Bronicki et al.	2000	29 children	RCT steroid vs placebo	Dexamethasone	1 mg/kg	IV	1-hour pre-CPB	eightfold decrease in interleukin-6 levels and a greater than a threefold decrease in tumour necrosis factor-α levels after CPB	less supplemental fluid during the first 48 hours, lower alveolar-arterial oxygen gradients during the first 24 hours, less mechanical ventilation	Benefit
Varan et al.	2002	30 children	RCT high-dose steroids vs low-dose steroid	Methylprednisolone	30 mg/kg vs 2 mg/kg	IV	30 minutes IV infusion pre-CPB	no difference in serum IL-6, IL-8, CRP and polymorphonuclear leukocyte counts	No effect	No benefit
Lindeberg et al.	2003	40 children	RCT steroid vs placebo	Dexamethasone	1 mg/kg	IV	post anaesthesia induction	a decrease in CRP but no change in von Willebrand factor antigen and S100B	No effect	No benefit
Schroeder et al	2003	29 children	RCT single dose steroid versus double dose	Methylprednisolone	30 mg/kg	IV	2 dose (4 hours before bypass and in bypass prime) vs 1 dose (intraoperative)	2 dose reduced myocardial mRNA expression for IL-6, MCP-1, and ICAM-1 both before and after bypass and had lower serum IL-6 and increased IL-10 at end-bypass vs 1 dose	combined steroid reduced fluid requirements, lower body temperature, and lower arteriovenous oxygen difference	Benefit
Checchia et al.	2003	28 children	RCT steroid vs placebo	Dexamethasone	1 mg/kg	IV	pre-CPB	not assessed	no effect	No benefit
Ando et al.	2005	20 neonates	RCT steroid vs placebo	Hydrocortisone sodium succinate after	0.18 mg . kg^{-1} for 3 days, 0.09 mg . kg^{-1} . hr^{-1} for 2 days and 0.045 mg . kg^{-1} for 2 days.	IV	post-CPB	not assessed	less fluid retention, better oxygenation and shorted ventilation times	Benefit

First author	Year of publication	Sample size	Study design	Type of steroid	Dose	Route	Regimen	Effect steroids on Inflammation	Effect steroids on clinical outcomes	Benefit/No Benefit/Harm
Amanullah et al.	2009	152 patients (1 month up to 18 years old)	RCT steroids vs placebo	Dexamethasone	1 mg/kg (maximum dose 12 mg)	IV	at the induction of anaesthesia – pre-operatively; at the time of initiation of CPB – intraoperatively; and 6 hours after the second dose – postoperatively	IL-6 was lower at 6 and 24 hours post-operatively and IL-10 levels were higher 6 hours post-operatively in the steroid group	No effect	No benefit
Graham et al.	2011	76 neonates	RCT single dose steroid vs double dose steroid	Methylprednisolone	30 mg/kg	IV and prime	8 hours pre-CPB, IV and in prime versus 8 hours preoperative only	preoperative interleukin-6 was reduced by 2-fold in the 2-dose steroid group	two-dose methylprednisolone was associated with higher serum creatinine and poorer postoperative diuresis	Harm
Heying et al.	2012	20 neonates		Dexamethasone	1 mg/kg	IV	4 hours pre-CPB	a decrease in myocardial expression of IL-6, IL-8, IL-1β, and TNF-α messenger RNA and decrease in protein synthesis of TNF-α; serum IL-6 significantly lower, IL-10 significantly higher in steroid-treated patients; lipopolysaccharide-binding protein significantly higher postoperatively pretreated patients	steroid recipients had a lower dobutamine requirement	Benefit
Keski-Nisula et al.	2013	40 neonates	RCT steroid vs placebo	Methylprednisolone	30 mg/kg	IV	after induction of anaesthesia	a decrease in IL-6 and IL-8 and raised levels of anti-inflammatory IL-10 in steroid-treated patient	blood glucose higher in the steroid-treated patient	No benefit
Keski-Nisula et al.	2015	45 children	RCT IV steroid vs in prime steroid vs placebo	Methylprednisolone	30 mg/kg	IV vs prime	after induction versus in prime	patients receiving steroids at induction had lower IL8 levels on weaning and 6 hours post-CPB versus placebo	blood glucose higher in the steroid-treated patient	No benefit
Graham et al.	2019	190 neonates	RCT steroid vs placebo	Methylprednisolone	30 mg/kg	IV	after induction of anaesthesia	not assessed	overall, no significant difference in the primary endpoint of the study (composite) death, mechanical circulatory support, cardiac arrest, hepatic injury, renal injury, or rising lactate level (>5 mmol/l) but benefit for palliative procedures	Benefit (palliative procedure)

Another larger RCT of 1200 neonates is currently recruiting patients https://clinicaltrials.gov/ct2/show/NCT03229538.

Some studies measured the effect of glucocorticoids on markers of myocardial injury (P. a Checchia et al. 2003; Malagon et al. 2005; Heying et al. 2012; Keski-Nisula et al. 2013; 2015; Pesonen et al. 2017) However, the markers of myocardial injury during cardiac surgery are difficult to interpret and correlate to the effect on clinical outcomes (see Table 3). If we look at the adult population, the large SIRS trial demonstrated that the steroid arm had an increase in myocardial injury measured by elevation of the CK-MB enzyme (Whitlock et al. 2015).

Given the limitations in the existing RCT evidence discussed above, the available meta-analyses should be interpreted with caution. The Cochrane meta-analysis by Robertson-Malt and colleagues demonstrated that corticosteroids do not significantly reduce postoperative complications as measured by the length of stay in ICU, peak core temperature and duration of ventilation (Robertson-Malt and El Barbary 2008). The most recent meta-analysis by Scrascia et al. demonstrated no significant effect of corticosteroids on mortality, mechanical ventilation time or ICU length of stay. Based only on 15 patients, the authors found a reduced prevalence of renal dysfunction associated with the use of steroid (13 versus 2 patients) (Scrascia et al. 2014).

Despite the small-sized randomized trials, there are several large observational studies in children that are worthy of discussion here (Table 4). The Pasquali et al. (Pasquali et al. 2012; 2010) studies provide us with the largest sample despite their retrospective design. In a large registry database study of 46730 children, of which 54% received perioperative corticosteroids, there was no difference in mortality or ventilation times; however, steroid use was associated with increased length of stay, more infection and greater use of insulin. In the analysis stratified by a congenital cardiac risk score (the RACHS-1 score), the increased

morbidity associated with steroid use was more evident in the lower risk categories (e.g. 1 to 3) (Pasquali et al. 2010). Later, the same group published a multicentre database study focused on 3180 neonates and found corticosteroids to be associated with increased infection across all regimens in the lower-risk groups. (Pasquali et al. 2012). A smaller observational study by Mastropietro et al. on 76 children undergoing complex heart surgery found that greater cumulative duration of steroid administration is associated with postoperative infection (Mastropietro et al. 2013).

Indeed, neonates were the focus of multiple studies because they are considered to be the most vulnerable to the CPB insult due to both the immaturity of the HPA axis (Green and Koch 2012) but because they also appear to display a distinct inflammatory response compared to older age groups (Alcaraz et al. 2002). A recent best evidence topic review by our group could not find a clear clinical benefit in steroid use mainly due to the limitations in the available evidence, earlier discussed (D. Fudulu et al. 2016).

Dreher et al. (Dreher et al. 2015) in a retrospective single centre database study, compared the effect of methylprednisolone administration in 303 children undergoing heart surgery during the six months before discontinuation of steroid use with a cohort of 222 children were no steroid was used. Overall, the steroid group had more wound infection and more respiratory failure requiring tracheostomy. There were no differences in the rest of the clinical outcomes (early mortality, ventilation time, renal failure, etc.). In the neonate subgroup analyses, no difference in clinical outcomes was detected. Using datasets from a clinical trial (the Paediatric Heart Network's Single Ventricle Reconstruction trial), on neonates that had the Norwood procedure, Elhoff et al. (Elhoff et al. 2016) compared the effect of steroid administration in 498 intraoperative steroid recipients with 51 non-recipients. In the multivariate analysis, the authors found no effect of steroid on lengths of stay but a trend towards better hospital survival in steroid non-recipients. This is one of the few studies where

the authors have also looked at the neurodevelopment outcomes at 14 months and found no difference. A significant limitation in the current studies of the impact of corticosteroids on the clinical outcomes paediatric heart surgery is the lack of long-term follow-up of the effect of early steroid administration on late neurocognitive outcomes. In other patient groups (preterm infants), the early administration of corticosteroids had detrimental effects. In an RCT of 262 infants with severe respiratory distress syndrome requiring mechanical ventilation, dexamethasone 0.25 mg/kg given every 12 hours for one week was associated with adverse effects on the neuromotor and cognitive function at school age. (Yeh et al. 2004). However, we must acknowledge that the findings of this study cannot be fully extrapolated to the paediatric surgical population, because these pre-term infants received glucocorticoids during a different stage in brain development as compared to term infants or older children where steroids can be less detrimental (e.g. not administered during the critical period of brain development).

In conclusion, most of the studies demonstrated that corticosteroids could dampen the systemic inflammatory response to surgery; however, the correlation to the effect on clinical outcomes remains unclear. The effect of corticosteroids on clinical outcomes was studied in several small-sized randomised controlled trials. This evidence is conflicting, with some studies showing a beneficial effect on clinical outcomes such as ventilation, oxygenation or renal function parameters while in other, the use of corticosteroids had no effect at all. Apart from being small sample-sized, these studies used various types of steroid and regimens; therefore, it is difficult to draw any valid conclusions. A few large registry studies raised concerns about the association of steroid administration with infection; however, their results remain limited by their retrospective design.

Table 3 Studies of the effect of steroids on markers of myocardial injury.

First author	Publication Year	Sample size	Study design	Type of steroid	Dose mg/kg	Route	Regimen	Cardiac marker	Effect on myocardial injury markers	Effect on Clinical outcomes	Cardioprotective
Checchia et al.	2003	28 children	RCT steroid vs placebo	Dexamethasone	1 mg/kg	IV	1-hour pre-CPB	cardiac troponin I (cTnI)	cTnI reduces 24 hours postoperatively in the dexamethasone group	No effect	Yes
Malagon et al.	2005	RCT steroid vs placebo	RCT steroid vs placebo	Dexamethasone	1 mg/kg	IV	during anaesthesia induction	cardiac troponin T (cTnT)	decrease in cTnT 8 h after admission	No effect	Yes
Heying et al.	2012	RCT steroid vs placebo	20 neonates	Dexamethasone	1 mg/kg	IV	4-hour pre-CPB	cardiac troponin T (cTnT)	cTnT lower in patients dexamethasone treated patients, 1 hour postoperatively	Lower dobutamine requirement	Yes
Keski-Nisula et al.	2013	40 neonates	RCT steroid vs placebo	Methylprednisolone	30 mg/kg	IV	after anaesthesia induction of anaesthesia	cardiac troponin T (cTnT)	No effect	No effect	No
Keski-Nisula et al.	2015	45 children	RCT IV steroid vs in prime steroid vs placebo	Methylprednisolone	30 mg/kg	IV vs prime	after anaesthesia induction versus in prime	cardiac troponin T (cTnT)	cTnT lower in patients in both at induction and in prime steroid recipients versus placebo, 6 hours post-CPB wean	higher blood glucose in both steroid-treated groups	No
Pesonen et al	2017	45 children	RCT of steroid IV vs in prime vs placebo	methylprednisolone	30 mg/kg	IV vs prime	after induction versus in prime	Plasma heart-type fatty-acid-binding protein and TnT	Plasma heart-type fatty-acid-binding protein and TnT decreased 6 hours post-op in both steroid regimens	No effect	Yes

Table 4Retrospective studies of the effect of steroids on clinical outcomes

First author	Year of publication	Sample size	Study design	Type of steroid	Dose	Route	Regimen/timing	Effect steroids on Inflammation	Effect steroids on clinical outcomes	Benefit/No Benefit/Harm
Pasquali et al.	2010	46 730 children	Retrospective,	variable ((methylprednisolone, prednisolone, dexamethasone, or hydrocortisone)	no data	no data	on the day before or day of surgery	Not assessed	Corticosteroids were associated with longer length of stay, greater infection, greater use of insulin, increased morbidity was most prominent in RACHS-1 categories 1 through 3 (lower risk groups)	Harm
Pasquali et al.	2011	3180 neonates	Retrospective,	methylprednisolone	no data	no data	methylprednisolone on the day before surgery and day of surgery, day of surgery only and day before surgery only	Not assessed	in a lower surgical risk group, there was a significant association of methylprednisolone with infection consistent across all regimens	Harm
Mastropietro et al.	2013	76 children	Retrospective	methylprednisolone,	all methylprednisolone 30 mg/kg, hydrocortisone 1 mg/kg (48%), dexamethasone 0.5 mg/kg (86%)	IV	methylprednisolone before surgical incision, hydrocortisone 6 hours for hemodynamic instability, periextubation dexamethasone every 6 hours	Not assessed	greater cumulative duration of corticosteroid exposure was independently associated with postoperative infection	Harm
Dreher et al.	2015	303 children vs 222 children)	Retrospective (non-steroid cohort versus steroid cohort, 6 months prior steroid discontinuation)	methylprednisolone,	30 mg/kg up to a maximum dose of 500 mg	in prime	in prime	Not assessed	steroids group had more postoperative wound infection and respiratory failure requiring tracheostomy	Harm
Elhoff et al.	2016	549 neonates	Retrospective	no data	no data	no data	intraoperative	Not assessed	improved hospital survival in the non-steroid group	Harm

10.1.3 Relative Adrenal Insufficiency

Another justification for the use of corticosteroids is the so-called *relative adrenal insufficiency* or *adrenal cortex exhaustion*, originally described during critical illness or sepsis. According to this definition, the concentration of plasma cortisol is not high enough to cope with the stress of surgery (Nair and Bonneau 2006; Annane 2000). Therefore, it is thought that perioperative steroid supplementation could cover for this potential deficit. However, defining relative adrenal insufficiency during critical illness remains a matter of debate, and very few studies have attempted to characterise the HPA axis physiology during surgery or critical illness in children. The most commonly used diagnostic criteria are the ACTH stimulation test described by Annane et al. (Annane 2000) In this test, synthetic ACTH (e.g. tetracosactrin) is given intravenously (Table 5). Cortisol is measured before and after the ACTH injection at 30 and 60 minutes. Plasma cortisol was measured by enzyme-linked fluorescent assay. In 189 consecutive adult patients with septic shock, the authors found that an incremental cortisol response to ACTH (defined as the difference between the basal cortisol and the highest value between cortisol measured at 30 minutes and 60 minutes) of < 9 µg/dL (248.2 nmol/L) or a high baseline cortisol concentration (>34 µg/dL or >937.9 nmol/L) were of good prognostic value for identifying patients at risk of death (Annane 2000). However, this test is based on a few time-point value measurements of cortisol. Therefore, this could prove inaccurate in the context of a dynamic, pulsatile cortisol secretion that was described in both healthy volunteers or adults undergoing heart surgery (Ben Gibbison et al. 2015; R. C. Bhake et al. 2013). The major limitation of the current literature aimed at understanding the HPA axis function during pediatric heart surgery, trying to correlate the findings of the ACTH test with the measures of clinical outcome. Other limitations include the measurement of only a few, random plasma

cortisol levels, the variability in the dose use for ACTH testing and most importantly the concomitant use of glucocorticoids at induction that obscures the assessment of the HPA axis (Gajarski et al. 2010; Garcia et al. 2010; Mackie et al. 2011; Wald et al. 2011b; Graham and Bradley 2017; Crawford et al. 2017; Teagarden and Mastropietro 2016; Maeda et al. 2016b; Mastropietro et al. 2014; Crow et al. 2014b; Bangalore et al. 2014; Schiller et al. 2013; Verweij et al. 2012; Sasser et al. 2012; Green and Koch 2012).

Kucera et al. measured the plasma cortisol at 6-time points in 24 children of various ages (ranging from 2 months to 15 years): the day before surgery, at the end of surface cooling, at the lowest temperature during CPB, 10 minutes after rewarming, at the end of CPB and on the 8th day, postoperatively (Kucera, Hampl, and Stárka 1986). Serum cortisol was measured using radioimmunoassay, and the haematocrit was determined in each sample so that the cortisol was corrected to the actual haemodilution. They found the cortisol levels to be in the range of the normal laboratory values with a trend toward increased during rewarming. Anand et al. measured cortisol levels at 7 timepoints, in 15 neonates: pre-operatively, pre-CPB, during DHCA, at 6 hours, 12 hours and at 24 hours. Plasma cortisol was measured using radioimmunoassay techniques. The peaks in cortisol secretion were recorded before CPB, and at the end of the operation, then, the cortisol levels fell below preoperative values at 12 hours (Anand, Hansen, and Hickey 1990). Gajarski et al. measured cortisol at ten time-points in 58 children: baseline - preoperative, time of surgery and every 6 hours up to 48 hours. They have stratified the cortisol profiles by patient groups: control (non-bypass or non-cardiac surgery procedures), CPB only (no DHCA), DHCA (no steroid used), and DHCA with steroid administration. The cortisol and ACTH peaked within 2 hours of surgery but without differences between the groups. In 9 patients, not from the DHCA-steroid group, they noted an elevated ACTH-cortisol ratio that correlated with an elevated inotropic score postoperatively

(Gajarski et al. 2010). Plasma cortisol was measured using a radioimmunoassay kit. In 21 neonates undergoing heart surgery with the use of CPB, Garcia and colleagues (Garcia et al. 2010) assessed the HPA axis with a low dose ACTH stimulation test (1 µg) on day one postoperatively. The patients that had a significant serum cortisol increase after ACTH test (>50 µg/dL or >1379.3 nmol/L) also had a higher mean arterial blood pressure at 48 hours postoperatively. Serum cortisol levels were measured with a standardised chemiluminescent immunoassay. All patients included in the study had dexamethasone 0.5 mg/kg midnight before surgery and at induction. In 38 neonates undergoing complex heart surgery, Mackie et al. measured serum cortisol at 3 time-points: preoperatively, at 24 hours and 48 hours postoperatively. They found the higher cortisol levels to be associated with greater atrial filling pressure and lower cardiac index. Again, all patients received methylprednisolone (30mg/kg at induction) (Mackie et al. 2011). Wald et al. (Wald et al. 2011a), in 51 children undergoing surgery with the use of CPB, measured total cortisol and corticosteroid-binding globulin levels pre- and postoperatively and after an ACTH test (subjects bellow two years received 125 µg while subjects > 2 years received 250 µg). The decreased CBG and increased free cortisol (> 6 µg/ml or >165.5 nmol/L) correlated with worse clinical outcomes (longer length of stay, longer ventilation time, increased fluid requirement). Total serum cortisol was measured using a chemiluminescent immunoassay. The authors found that only 17.6% of the patient had a low baseline total cortisol and all had a normal ACTH stimulation test. They concluded that total cortisol is not a good measure of HPA axis function in this setting. All patients received 1 mg/kg of dexamethasone before CPB. Verweij et al.(Verweij et al. 2012) in a retrospective analysis of 62 patients with low cardiac output post paediatric heart surgery, found no effect of hydrocortisone administration in patients with insufficiency (defined as having basal plasma cortisol was <100 nmol/l). Sasser et al. (Sasser et al. 2012) in a retrospective analysis of 41 neonates found that a postoperative serum cortisol level >10 µg/dL (275.8 nmol/L) is not

associated with worse clinical outcomes (lactate measurements, inotropic score, fluid requirement, arteriovenous saturation difference, mean blood pressure, mean CV, mean heart rate or ventilation time) and no difference in steroid responsiveness compared to the cortisol > 10 µg/dL (275.8 nmol/L) group . Schiller et al.(Schiller et al. 2013) in a prospective analysis of 119 children undergoing heart surgery measured cortisol levels pre-operatively and at 18 hours after surgery. The authors defined adrenal insufficiency as a measured postoperative cortisol level of less than 18.1 µg/dL (499.3 nmol/L) or a delta cortisol (e.g. the difference between postoperative cortisol and preoperative cortisol measurements) value of < 9 µg/dL (248.2 nmol/L). Plasma cortisol was measured using a solid-phase competitive chemiluminescent enzyme immunoassay. There was no significant correlation between patients that had adrenal insufficiency and the procedure complexity (low or high). Furthermore, the postoperative course (ICU stay ventilation time, lactate, urine output) of children with adrenal insufficiency, was not different from the ones without adrenal insufficiency. All patients included had methylprednisolone (30mg/kg) at induction. A study by Bangalore and colleagues (Bangalore et al. 2014) assessed postoperative serum cortisol levels at three time-points: immediately after surgery and in the first and second postoperative mornings. Serum cortisol was measured using a competitive immunoassay. The cortisol fell significantly over the first 24 hours. Higher postoperative cortisol measurements were associated with increased morbidity. All patient had methylprednisolone (30mg/kg) at induction. Crow et al.(Crow et al. 2014a) measured serum cortisol, and dexamethasone blood levels after 1 mg/kg of dexamethasone were administered pre-CPB in 32 infants undergoing cardiac surgery. Blood cortisol was measured by liquid chromatography-tandem mass spectrometry. They noted significant variability in dexamethasone levels and grouped the patients into high dexamethasone (>15 µg/dL or 413.7 nmol/L) or low dexamethasone (<15 µg/dL or 413.7 nmol/L) based on their level at ICU arrival. Although the patients that had

higher dexamethasone levels had more pronounced suppression of cortisol levels postoperatively compared to basal levels, this did not translate into any impact on clinical outcomes. Teagarden et al. worked on 24 patients that underwent surgery for congenital heart disease(Teagarden and Mastropietro 2016) found that lower pre-hydrocortisone cortisol was associated with improved haemodynamics after hydrocortisone administration. Serum cortisol was measured using a radioimmunoassay technique. Maeda et al. (Maeda et al. 2016a) classified 32 neonates undergoing heart surgery into patients with adrenal insufficiency (baseline cortisol < 15 µg/dL (413.7 nmol/L) or incremental increase after testing of <9 µg/dL (248.2 nmol/L) and baseline total cortisol of 15-34 µg/dL or 413.7-937.9 nmol/L) and a group with normal adrenal function, after ACTH test (3.5 µg/kg of tetracosactide acetate). All patients received 1mg/kg hydrocortisone perioperatively every 6 hours up to 18 hours from first hydrocortisone dose. Only the patients diagnosed with adrenal insufficiency exhibited a significant increase in mean blood pressure and urine output as response to hydrocortisone administration. A recent study by Crawford et al. (Crawford et al. 2017) was aimed to correlate relative adrenal insufficiency to clinical outcomes in 40 neonates undergoing complex heart surgery. Like the studies discussed above, all patients received preoperative methylprednisolone. The authors defined adrenal insufficiency as <9 µg/ml (248.2 nmol/L) increase in cortisol at 30 min post ACTH test (cosyntropin 1 mcg). Serum cortisol was measured using an immunoassay. Five per cent of the patients had adrenal insufficiency post-CPB, and this was significantly associated with increased serum lactate and higher inotrope requirement.

We still do not have an accepted definition for adrenal insufficiency in children undergoing heart surgery; hence it is difficult to draw conclusions about the effect of corticosteroids on the adrenal function and clinical outcomes. Most of the studies so far used very few time-point measurements and conflicting results and were undertaken on children that

received corticosteroids at induction, therefore, making an accurate assessment of the HPA axis almost impossible.

Another question that arises from this literature review is what the best method of measuring cortisol is? Most of the studies have used a radioimmunoassay method. One limitation of this technique is the use of specific antibodies that can cross-react with other endogenous or exogenous steroid, thus providing inaccurate. Furthermore, the current assays that measure total cortisol (bound and unbound). This implies that any conditions that affect the concentration of the binding proteins can provide inaccurate results. For example, cortisol concentration can be falsely low in liver conditions or very unwell patients that are associated with low binding protein concentration. On the other hand, pregnancy or use of oral contraceptives can result in a higher cortisol concentration. The mass methods such as gas chromatography-mass spectrometry (GC-MS) or LC-MS (liquid chromatography-mass spectrometry) outperform radioimmunoassay methods in terms of specificity and sensitivity (El-Farhan, Rees, and Evans 2017)

Table 5 Studies aimed at understanding the hypothalamic-pituitary adrenal axis function in children.

First author	Year	Sample size	Study design	Steroids perioperatively	Cortisol timepoint	Frequency of cortisol measurement	ACTH test	ACTH test dose	Definition of adrenal insufficiency used in the study	Correlation to clinical outcome	Main finding
Kucera et al	1986	24 children	Observational	no data	6	1 time-point day before surgery, 4 time-points day of surgery and 1 timepoint 8th day of surgery	no	NA	none	not assessed	cortisol levels to be in the range of the normal laboratory values with a slight increase during rewarming
Anand et al	1990	15 neonates	Observational	no	7	pre-op, pre-CPB, during DHCA, end of the operation, 6 hours, 12 hours and 24 hours.	no	NA	none	survival rate	non-survivors (n=4) had higher concentrations of cortisol than survivors
Gajarski et al.	2010	58 children	Observational	10 patients in the DHCA arrest group	10	before surgery, after surgery, 6, 12, 18, 24, 30, 36, 42 and 48 hours	ACTH measured at same time points with cortisol	NA	ACTH/ serum cortisol ratio cut-off of >15	Peak cortisol level did not correlate with simultaneous inotrope score; nine patients had increased ACTH/cortisol ratios in association with elevated inotrope requirement (none of these patients had steroids)	cortisol peaked within 2 hrs of surgery; ACTH inversely correlated with bypass time in patients with DHCA but not with circulatory arrest time
Garcia et al.	2010	21 neonates	Retrospective	All patient's dexamethasone 0.5 mg/kg before surgery	2	basal and post ACTH test	ACTH test, first post-op day in patients with worsening haemodyna mic status	1 μg	basal serum cortisol level ≤ 20 mg/dL and a post-stimulation serum cortisol increase ≥ 9 mg/dL	all neonates with haemodynamic instability had low basal serum cortisol; 48 hours post-surgery the mean arterial pressure in the groups with a serum cortisol increase after ACTH stimulation (<30 mg/dL vs>50 mg/dL) was significantly different	cortisol level cut-off of ≤20 mg/dL may not be applicable in neonates undergoing heart surgery
Mackie et al.	2011	38 neonates	Observational	all patients had Methylprednisolo ne, 30 mg/kg, IV at anaesthetic induction	3	preoperative, at 24 hours and 48 hours post-surgery	no	NA	none	Higher cortisol levels were associated with greater atrial filling pressures and a lower cardiac index	cortisol levels were low in most subjects
Wald et al	2011	52 children	Observational	all patients had received 1 mg/kg dexamethasone before CPB, not to exceed a 10-mg total dose	2	pre - and postoperative	no	cosyntropin: 250 μg for children >2 yrs. of age and 125 μg for children < 2 years	reference range for normal total plasma cortisol was 3–21 μg/dL. Free cortisol in critical illness was defined as >2.0 g/dL. A normal free cortisol value after cosyntropin test was defined as > 3.1 g/dL and total serum cortisol increase ≥ 9 mg/dL	nine patient had low total cortisol (<3 μg/dL) baseline but normal ACTH stimulation test). Patient with free cortisol increase difference of > 6 μg/dL had a longer length of stay, higher inotrope scores, greater fluid requirement, longer ventilator times	using total cortisol to investigate adrenal dysfunction may be inadequate, decreased corticosteroid binding globulin levels post-stimulation associated with worse clinical outcomes

First author	Year	Sample size	Study design	Steroids perioperatively	Cortisol timepoint	Frequency of cortisol measurement	ACTH test	ACTH test dose	Definition of adrenal insufficiency used in the study	Correlation to clinical outcome	Main finding
Verweij et al.	2012	62 children with low cardiac output	Retrospective	all patients dexamethasone 0.5 mg/kg before surgery	1	basal cortisol	no	NA	bassline value of total cortisol of <100 nmol/l and	a similar effect of hydrocortisone in the groups with low or normal basal cortisol levels	baseline value of total cortisol of <100 nmol/l not adequate to define adrenal insufficiency
Schiller et al.	2013	119 children	Observational	all patients received intravenous methylprednisolone, 30 mg/kg to a maximum dose of 300 mg/kg, at induction	2	before and 18 hours after surgery	no	NA	postoperative cortisol level <18.1 µg/dL or delta cortisol (postoperative cortisol - preoperative cortisol) <9 µg/dL	normal adrenal function (NAF) subgroup had greater inotropic support at 12, 24, and 36 h after surgery and a higher lactate level at 12 and 24 h after surgery; no differences in outcomes between patients with adrenal insufficiency and normal adrenal function in the first 36 hours, no correlation between adrenal insufficiency and procedure complexity	adrenal insufficiency does not translate into worse clinical outcomes
Bangalore et al.	2014	33 neonates	Observational	all patients methylprednisolone, 20 mg/kg, at induction	3	day 0 (after intensive care unit admission); day 1 (first morning of surgery), day 2 (second morning of surgery)	no	NA	basal cortisol of less than 16 µg/dL, with a change in cortisol of less than 9 µg/dL after cosyntropin	Higher cortisol was associated with greater morbidity, including the need for preoperative ventilation, increased total duration of ventilation, duration of inotropic support, and hospital length of stay	high postoperative cortisol was associated with increased postoperative morbidity
Crow at al	2014	32 infants	Observational	1 mg/kg of dexamethasone before CPB initiation.	7	after anaesthesia induction, after CPB, after intensive care unit (ICU) arrival, and 4, 8, 12 and 24 hours after surgery	ACTH measured at same time points with cortisol	NA	NA	no difference in clinical outcomes between patients with high dexamethasone levels (≥ 15 mg/dL) and low dexamethasone levels (≤ 15 mg/dL); cortisol levels remained low throughout the first 24 postoperative hours even after dexamethasone levels neared zero	dexamethasone levels are highly variable despite a standardised administration protocol
Teagarden et al.	2016	24 patients < 21 years	Retrospective	intraoperative dose of methylprednisolone (30mg/kg) before surgical incision and 1mg/kg intravenously every 6 hours for patients with haemodynamic instability	1	pre-hydrocortisone treatment serum cortisol	no	NA	Favourable responders were defined as patients in whom, at 24 hours after hydrocortisone initiation, either (1) systolic blood pressure was increased or unchanged and vasoactive-inotrope score was decreased or (2) systolic blood pressure increased by ⩾10% of baseline and the vasoactive-inotrope score was unchanged	serum cortisol obtained before initiation of hydrocortisone was significantly lower in patients who responded favourably	total serum cortisol may, therefore, be helpful in identifying children recovering from cardiac surgery who may or may not haemodynamically improve with hydrocortisone

First author	Year	Sample size	Study design	Steroids perioperatively	Cortisol timepoint	Frequency of cortisol measurement	ACTH test	ACTH test dose	Definition of adrenal insufficiency used in the study	Correlation to clinical outcome	Main finding
Maeda et al.	2016	32 neonates	Retrospective	Hydrocortisone 1 mg/kg, was given every 6 hours immediately after ACTH test	3	baseline and at 30 and 60 minutes after the tetracosactide stimulation	yes	3.5 μg/kg of tetracosactide acetate	baseline cortisol <15 μg/dL or incremental increase after testing of <9 μg/dL	one-fifth of infants developed adrenal insufficiency, steroid administration in these patients resulted in a significant increase in blood pressure and urine output	steroid replacement therapy improved hemodynamic only in the subgroup with adrenal insufficiency
Crawford et al.		40 neonates	Retrospective	methylprednisolone 10mg/kg 8 h and 1 h		basal and 30 minutes post ACTH test	ACTH measured at same time points with cortisol	1 μg cosyntropin the day before surgery before preoperative methylprednisolone; and the second 1 h after separation from CPB	AI was defined as <9 μg/dL increase in cortisol at 30 min post ACTH test	32.5% had adrenal insufficiency post CPB, AI was associated with increased median colloid resuscitation, higher serum lactate	adrenal insufficiency determined by a low dose ACTH test occurs in one-third of the patients is not affected by pre-operative steroid administration

10.1.4 Corticosteroids and cerebral protection

Cardiac surgery with the use of deep hypothermic circulatory arrest[2] is known to be associated with impaired cerebral oxygen metabolism and cerebral oedema (Langley et al. 2000; Greeley et al. 1991). Corticosteroids are used to treat cerebral oedema secondary to brain tumours (Koehler 1995) but are were previously used in the context of head trauma (Olldashi et al. 2004). Therefore, by extrapolation, another justification for the use of corticosteroids in deep hypothermic circulatory arrest (DHCA) surgery. However, if we look at the large CRASH trial, this showed an increase in death at two weeks and six months with steroid use (Baigent et al. 2005; Olldashi et al. 2004). The evidence of the impact of corticosteroids on brain protection during the use of DHCA has not been studied extensively. This evidence is limited to *in vitro* studies (Schmitt et al. 2006) and *in vivo* experiments on piglets (Schubert et al. 2005; Langley et al. 2000) and the results are quite conflictive.

Schmitt and colleagues investigated the effect of deep hypothermic circulatory arrest on an *in vitro* model of mouse neonatal astrocytes, neurons and BV-2 microglia cells. The effect of methylprednisolone (100 nM) was tested in cells that were incubated according to a protocol that mimics the temperature changes during paediatric deep hypothermic circulatory arrest: deep hypothermia, slow rewarming and normothermia. The authors measured in all cell lines the cytotoxicity and the production of IL-6 as a marker for neuroprotection and regeneration. While steroid administration did not affect the normothermic treated cells, in the

[2] Deep hypothermic circulatory arrest (DHCA) is a surgical technique that involves cooling the body temperature (with the help of the CPB machine) to 14 to 20 degrees Celsius followed by complete cessation of the circulation for up to approximately 1 hour to allow certain operative steps.

deep hypothermia treated cells methylprednisolone increased cell survival but decreased the protective IL-6 (Schmitt et al. 2006).

Langley et al. randomised two groups of 8 piglets to placebo and methylprednisolone 30 mg/kg, intramuscularly, given 8h and 2 hours before induction. All piglets underwent cooling to 18^0C, 60 minutes of circulatory arrest and 60 minutes of reperfusion and rewarming. The steroid arm had a significantly higher recovery of the cerebral blood flow and cerebral oxygen metabolism (Langley et al. 2000). In a later study, Schubert and colleagues randomised two groups of 7 neonatal piglets to methylprednisolone (30 mg/kg), intravenous, given earlier, at 24 hours before surgery and placebo. The piglets were then cooled to a lower temperature - 15^0C and had a more extended period of DHCA – 120 minutes and rewarming for 40 minutes. The authors conducted quantitative histological studies in the various brain regions: hippocampus, cortex, cerebellum and caudate nucleus. The steroid arm had more neuronal cells death and neuronal apoptosis in the dentate gyrus and hippocampus.

The neuroprotective potential of corticosteroids in cases of paediatric heart surgery with the use of deep hypothermic circulatory arrest has been the least studied. The current evidence is contradictory, and the results from the available animal studies highlight the need for human studies aimed at this high-risk patient subgroup.

10.1.5 Conclusions

I found no clear evidence that the anti-inflammatory effect of corticosteroids does translate into better clinical outcomes. Most randomized studies in the literature report too few patients, different endpoints and use various steroid types, doses and regimens. A trial on neonates found a potential benefit of steroids in new-borns undergoing palliative procedures. Though some registry studies looked at the effect of corticosteroids on larger patient

populations, they are limited by their retrospective design. The effect of corticosteroids on clinical outcomes will need to be clarified by a large, multicentre, randomised controlled trial with clear agreed methodology and aims. Our knowledge about the basic physiology of the hypothalamic-pituitary axis during surgery remains limited, and it is unclear how to define adrenal insufficiency in the context of paediatric heart surgery and if corticosteroids do play a role. Such an understanding of the dynamic, pulsatile cortisol secretion can be gained from studies that use frequent time-point measurements in patients not receiving corticosteroids. Finally, the neuroprotective effect of corticosteroids during deep hypothermic circulatory arrest remains even more controversial and warrants further research

10.2 Corticosteroids and Other Anti-Inflammatory Strategies in Paediatric Heart Surgery: A National Survey of Practice

Sections 10.2.1 to 10.2.4 were largely taken from an original article published in the World Journal for Pediatric and Congenital Heart Surgery (D. P. Fudulu, Schadenberg, et al. 2018). The authors contributions were the following: Daniel Fudulu - conception and design of the analysis; collection of the data; the analysis and writing of the paper; Alvin Schadenberg - collection of the data, revision; Ben Gibbison -revision; Ian Jenkins - conception and design of the analysis; Stafford Lightman, Gianni Angelini - supervision, revision; Serban Stoica - supervision, revision and collection of the data.

10.2.1 Introduction

As seen in the previous chapter, there is a lack of evidence and even consensus on the utility of steroids for heart surgery with the use of CPB. This lack of consensus was also reflected locally, in the Bristol Children's Hospital, which was the first recruitment centre for the Peacock study (see next chapter). Some of the consultants were reluctant in omitting steroids for the patient recruited in the Peacock Study. Therefore, we decided to design a national survey to assess current preferences consultant anaesthetists in the UK to administer steroids in paediatric heart surgery with the use of CPB. As a secondary objective, we also surveyed the use of other anti-inflammatory strategies including modified ultrafiltration, aprotinin and heparin-coated circuits.

10.2.2 Material and Methods

A 19-question survey was distributed to the Congenital Cardiac Anaesthetic Network (CCAN) UK consultant email list (Figure 22). The responses were collected during December

2015-November 2016 period, and several iterations were conducted until an adequate response from all units was received. Results were collected using the Survey Monkey Inc. platform, San Mateo, California, USA and analysed using Graph Pad Prism version 7.00 for Windows, La Jolla California, USA.

1. Do you administer steroids for paediatric cardiac surgery involving cardiopulmonary bypass (CPB)?

Yes

No

2. Every case?

Yes

No

3. If "No", which of these WOULD you always give steroids?

Neonates

"Redos"

Deep hypothermic Circulatory Arrest

Other (please describe)

4. Drug name?

Dexamethasone

Hydrocortisone

Methylprednisolone

Other (please specify)

5. Multiple doses?

Yes

No (Single dose)

6. Timing of the dose?

Preoperatively

Pre-CPB (during induction of anaesthesia)

In prime

Post CPB

7. How many hours prior to CPB?

8. How many hour post-CPB?

9. Single dose in mg/kg?

10. Maximum dose in mg?

11. Multiple dose (describe regimen)?

12. Do you continue steroids after CPB?

Yes

No

13. If "Yes", describe dosing regimen:

14. Do you use modified ultrafiltration at conclusion of CPB?

Yes

No

15. If "Yes", in what range?

Up to 6 kg

Up to 10 kg

Over 10 kg

16. Do you use any other immunomodulatory therapies?

None

Intravenous immunoglobulin

Heparin-coated circuit

Aprotinin

Other (please specify)

17. If hyperglycaemia is seen (e.g. over 12mmol/l), do you routinely use an insulin infusion?

Yes

No

18. Institution?

19. Any other points or suggestions you would like to add:

Figure 22 Survey questions

10.2.3 Results

The survey was sent to 60 consultants from all 12 National Health Service (NHS) units across the UK and Ireland performing paediatric heart surgery. We received 37 responses (61.7%) with an average of 3 responses per centre (ranging from 1 to 5 responses). In 5 out of the 12 centres, the practice of administering steroids varied between consultants within that centre (Figure 23 B). Out of the 37 respondents from the UK and Ireland: 24 (64.8 %) reported the use of CS while 13 (35.1%) do not use CS at all. Seven (7/24, 29.1%) anaesthetists administer CS in every case while 17 (17/24, 70.8%) in selected cases only (Figure 23 A).

There were 29 indications cited for steroid use in total, ranging from 1 to 6 indications per respondent (n=17). The most common indications for corticosteroid administration was surgery in neonates (9, 31%), surgery with use of DHCA (9, 31%), re-do cases (2, 6.9%), the Norwood operation (2, 6.9%), high perioperative inotrope requirement (2, 6.9%), complex atrial/ventricular septal defects (2, 6.9%), switch operation (1, 3.4%), long CPB time (1, 3.4%) and surgery in infants (1, 3.4%)

The most widely used corticosteroid was dexamethasone; used by 17 consultants (17/24, 70.8%) followed by methylprednisolone used by 4 (4/24, 16.7%) and hydrocortisone used by 3 consultants (3/24, 12.5%). Almost all consultants (23/24, 95.8 %,) administer a single dose of steroid at induction, and only one (1/24, 4.2%) administers a two-dose regimen (dose at induction and one at 6 hours from the first dose). Dexamethasone doses ranged from 0.5mg/kg to 1 mg/kg, methylprednisolone dose ranged from 20mg/kg to 30mg/kg, and hydrocortisone was administered at a dose of 4mg/kg. If we calculate the equivalent anti-inflammatory dose of dexamethasone for the rest of CS administered, there is a further variation with dexamethasone doses ranging from 0.15 mg/kg up to 5.62 mg/kg (Table 6).

Most consultants (24/37, 65.9%) use modified ultrafiltration[3] after CPB. Seven consultants (7/24, 29.25%) use ultrafiltration in children up to 6kg, 13 (13/24, 54.2%) in children up to 10 kg and 4 consultants (4/24, 16.7%) in children over 10 kg. Out of the 32 respondents: 15 (15/32, 46.9%) consultants use aprotinin and only 3 (3/24, 9.4%) heparin-coated circuits.

[3] Modified ultrafiltration is a technique that tackles the capillary leak syndrome associated with the paediatric cardiopulmonary bypass and that results in increased content of water in the tissue. The technique is applied immediately after cessation of the CPB and aims to ultrafilter and haemoconcentrate patients to improve outcomes (Eliott 1993).

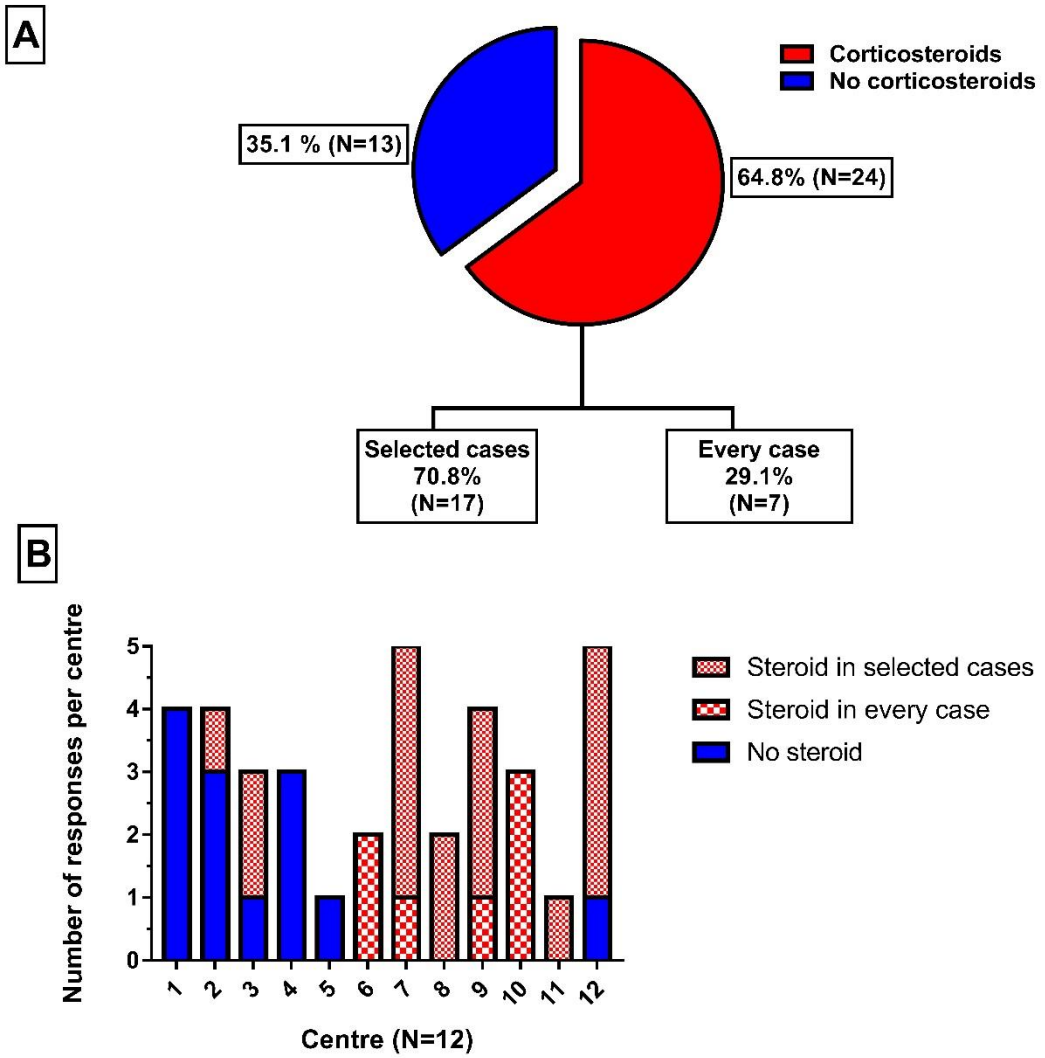

Figure 23 Corticosteroid preferences (A) variation within and between centres (1B)

Corticosteroid	N	%	Dose range (mg/kg)	Dexamethasone equivalence (mg/kg)
Dexamethasone	17	71%	0.5 - 1	0.5 – 1
Methylprednisolone	4	17%	20 - 30	4 -6
Hydrocortisone	3	13%	4	0.2

Table 6 Glucocorticoid doses and equivalence

10.2.4 Discussion

Across the UK and Ireland, approximately 4500 paediatric heart operations involving CPB are performed every year("CCAD - Congenital Analysis - Summary Data - By Year" n.d.). Therefore, understanding the impact of steroids on clinical outcomes is a significant health issue. In contrast to previous surveys (Allen et al. 2009; P. A. Checchia et al. 2005), we have also assessed the preference of steroid administration not only *between* centres but also *within* centres. In the survey by Checchia et al. 36 responses were returned from each centre representative (P. A. Checchia et al. 2005), and in the survey, by Allen et al. (Allen et al. 2009) only 12 responses were returned in total from centre representatives. Neither study looked at variation within centres. In our survey, we found variation in steroid preferences within five centres. This suggests the lack of local consensus between consultants and a lack of protocols in individual centres.

More than half of consultant anaesthetists use CS in paediatric heart surgery (65%) in some form. However, we noticed a trend in reduced CS use compared to an international survey by Chechia et al.(P. A. Checchia et al. 2005) from 2005 (97%) and an older UK survey by Allen et al. (Allen et al. 2009) (80%). Out of the consultants that use CS, the majority administer them in selected cases only (71%) compared to 60% and 58% in the previous surveys of practice (P. A. Checchia et al. 2005; Allen et al. 2009). This trend towards reserving CS for selected cases reflects the ongoing lack of consensus and need for further evidence in the "high risk" patient groups.

We found variability in the indications of CS use. The most common indications for steroid use were surgery in neonates (31%) and use of DHCA (31%). Indeed, we know very little about the stress response in neonates. The immaturity of their HPA axis means they are less likely to cope with the stress of surgery (Green and Koch 2012). We conducted a recent systematic literature review focused on this group and found limited evidence (D. Fudulu et al.

2016). The use of CS for neuroprotection in cases with use DHCA is another unanswered question. The available studies assessing the effect on brain protection used piglet CPB models. A study by Langley et al. suggested cerebral protection if methylprednisolone (MP) is given early: 8 to 12 hours pre-operatively (Langley et al. 2000).

On the other hand, a study by Schubert et al. (Schubert et al. 2005) showed no benefit for MP 24-hour pre-treatment. Other indications for CS were aimed at the high-risk procedure groups. There are a few studies in this group, and the evidence is again conflicting. In a small RCT of 20 neonates undergoing arterial switch, pre-treatment with MP reduced the expression of myocardial and plasma cytokines that translated in lower inotrope requirement and decreased myocardial damage(Heying et al. 2012). A large retrospective analysis of 549 neonates who underwent the Norwood procedure found intraoperative steroid administration was not associated with improvement in outcome.

Furthermore, steroid non-recipients had better hospital survival but longer intensive care and hospital stays (Elhoff et al. 2016). With regards to inotrope requirements, there is some evidence that so-called “rescue” steroids improve hemodynamically and lower inotrope requirement (Neunhoeffer et al. 2015). In an RCT of 40 neonates by Roberto et al. the use of a prophylactic postoperative steroid infusion reduced inflammation, improved fluid balance and urine output and allowed a faster wean from catecholamines or vasopressin(Robert et al. 2015). Similar to our survey of prophylactic steroid administration, a recent survey by Flores et al. (Flores et al. 2017) found significant variability in the indications for corticosteroid administration in patients with severe low cardiac output syndrome.

We found variation in both the type of steroid used and in terms of the doses administered. This is a similar finding to previous surveys of practice (Allen et al. 2009; P. A. Checchia et al. 2005). Almost all consultants give one dose at anaesthesia induction, and only one reported to administer a two-dose regimen at induction and at 6 hours from the first dose.

A few studies on piglet CPB models found some benefit of early CS administration on pulmonary function (Lodge et al. 1999) and brain protection (Langley et al. 2000).

In contrast to US practice, where steroids are administered in the majority of the cases in the CPB prime solution (the fluid that is in the CPB prior to going on "bypass") (Ungerleider 2005), in our survey we noted that steroids are given most of the time pre-operatively, at anaesthetic induction. A three-arm RCT of 45 children evaluated the effect of methylprednisolone administration in CPB prime versus intravenously (at anaesthetic induction) during cardiac surgery. There was no difference in terms of clinical outcomes between the three groups. However, steroids given at induction were superior in terms of anti-inflammatory effect compared to the CPB prime route(Keski-Nisula et al. 2015).

Modified ultrafiltration use in paediatric heart surgery can remove the excess of water and inflammatory mediators during CPB. The impact on the postoperative course is haemoconcentration, reduced need for transfusions, and improvement of cardiac and respiratory function (Wang, Palanzo, and Ündar 2012). According to previous surveys, its use amongst paediatric heart surgery centres ranged from 75-80% (P. A. Checchia et al. 2005; Allen et al. 2009). In the current survey, fewer centres used MUF (e.g. 66%). This could be explained by the emergence of low prime volume extracorporeal circuits, avoidance of severe haemodilution or efficient use of conventional ultrafiltration that no longer justifies the use of MUF and its associated risks (Jonas 2017). Aprotinin is a potent anti-fibrinolytic but also has anti-inflammatory properties that could be advantageous in paediatric heart surgery. However, its safety profile in both adult and paediatric patients remains a matter of debate (Goobie 2014). This is reflected in the current survey, where about half of the consultants reported its use.

There are no studies to date investigating the combined effect of the various anti-inflammatory modalities. The strengths of this survey are the analysis of steroid variation within centres and of steroid indications for the various patient groups. The weakness is its

response rate of only 61.7 %. However, this compares to previous surveys on this topic (P. A. Checchia et al. 2005; Allen et al. 2009). Another source of bias could be steroid administration by other healthcare professionals, including surgeons, intensivists or perfusionists. However, within the UK, the prophylactic, perioperative steroid administration is usually governed by the paediatric cardiac anaesthetist.

The current survey offers the following observations. The use of prophylactic corticosteroids in paediatric heart surgery remains a matter of intense debate. We found variations in steroid administration both within centres and between centres. Most consultant anaesthetists give steroids in selected cases. However, there was heterogeneity in the cited indications, dose and type of steroid used. Of those that used steroids, almost all administer a single dose of CS preoperatively at induction. These results reflect the lack of evidence from placebo-controlled randomised trials of CS versus placebo, powered to look at the impact on clinical outcomes. In the context of the low mortality and morbidity associated with paediatric heart surgery, the obstacle to conducting such research is the recruitment of a sufficiently large sample size to detect any effect. This also implies that the effect size of corticosteroid use is small across a population or may be limited to selected pathologies. This survey also highlights the need to investigate the role of steroids in the "vulnerable" patient groups such as neonates or complex surgical cases with the use of deep hypothermic circulatory arrest.

11 THE PEACOCK STUDY – A DETAILED CHARACTERISATION OF THE HYPOTHALAMIC-PITUITARY ADRENAL PHYSIOLOGY DURING AND AFTER PAEDIATRIC HEART SURGERY

"There is no more difficult art to acquire than the art of observation, and for some men it is quite as difficult to record an observation in brief and plain language"

William Osler

11.1 Introduction

11.1.1 The knowledge gaps

There is little research into the basic physiology of the HPA axis in children undergoing heart surgery. As shown in the previous chapters, no study to date has examined ultradian rhythms and therefore, pulsatile patterns of cortisol in children. Because of this knowledge gap, there is an ongoing debate around the modulation of the HPA axis function perioperatively to improve outcomes. Surgery with CPB is a potent activator of the systemic inflammatory response and hence of the HPA axis. It is believed that this activation is augmented in the paediatric population (D. P. Fudulu, Gibbison, et al. 2018).

Our first difficulty in elucidating the role of steroids in paediatric CPB is our lack of understanding of the systemic inflammatory response to surgery and the difficulties of detecting a significant treatment effect in the context of a low mortality or morbidity results of contemporary paediatric heart surgery. As shown in the previous chapter, there is a lack of HPA axis physiology understanding. This stems from the difficulty of measuring plasma cortisol frequently enough due to the limitations of multiple blood samples taken from a small circulating volume - particularly in low weight babies. Indeed, an accurate assessment of the HPA axis requires frequent cortisol testing (Powell et al., 2017). Most of the studies to date

have measured cortisol at a few time points over long periods (D. P. Fudulu, Gibbison, et al. 2018). Another common limitation of these studies is that they have attempted to assess HPA axis function after potent corticosteroids have been given pre-operatively (Gajarski et al. 2010; Mackie et al. 2011; Bangalore et al. 2014; Maeda et al. 2016a; Garcia et al. 2010; Wald et al. 2011a; Schiller et al. 2013; Crawford et al. 2017; Verweij et al. 2012; Sasser et al. 2012; Teagarden and Mastropietro 2016; Anand, Hansen, and Hickey 1990) (Figure 24). Finally, defining abnormal HPA axis physiology by using limited points of cortisol or synthetic adreno-corticotropic-hormone stimulation tests is inaccurate in the context of the dynamic cortisol release.

The Peacock Study aimed to gain some understanding of the HPA axis physiology in children of various ages, not receiving perioperative steroids and undergoing various cardiac procedures. This was achieved by using a novel automated tissue microdialysis system that allows very frequent and cortisol measurements that allows the dynamic, pulsatile nature of the system to be seen. I this chapter I will discuss our methods, the study protocol and data from the 36 patients that so far have been recruited to the study.

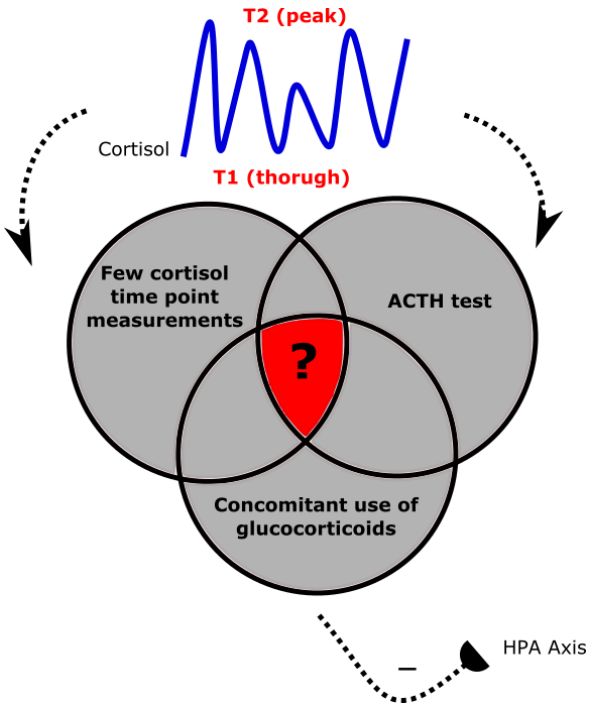

Figure 24 Limitations of our understanding of the paediatric HPA axis stress response to surgery in children

11.2 Hypothesis and aims

The following hypotheses were tested:

1. The use of an automated microdialysis system is a feasible method of measuring cortisol profiles in children;
2. Children display a dynamic, pulsatile cortisol response in response to surgery
3. The cortisol patterns vary according to the:
    a. Age of the child
    b. The underlying heart defect (e.g. cyanotic vs acyanotic defects)
    c. Pubertal state (pre- and post-pubertal)
    d. Type of procedure (open heart surgery with the use of CPB versus percutaneous cardiac procedures)

Furthermore, the aims of the thesis are reported as the following:

1. To assess the performance of an automated tissue microdialysis system in measuring cortisol in children undergoing heart surgery and cardiac procedures;
2. To measure tissue cortisol and cortisone profiles in children undergoing heart surgery and cardiac procedures recruited in the various patient groups set up in the study protocol;
3. To measure serum cortisol, ACTH, CBG, cytokines and sex hormones at the various time-points set up in the protocol;
4. To record various clinical outcomes for correlation with the hormone profiles (refer to Study Protocol Section).

11.3 Methods

11.3.1 Overview of the 24-hours automated microdialysis system structure

The principle of microdialysis involves the insertion of a small microdialysis catheter in the tissue that allows continuous monitoring of the tissue chemistry. A physiological, isotonic solution is slowly pumped through a semipermeable membrane and this solution is equilibrated with the surrounding tissue fluid (extracellular fluid). This allows an accurate measurement without the need o blood sampling.

The ideal measurement of cortisol measurement in children has to be acceptable for patients, parents and staff. Specifically, it must be reliable and automatic to facilitate a frequent cortisol measurement and must also have minimal impact on the circulating blood volume of the child. This is crucial, especially in neonates, that are in a critical state perioperatively and have blood taken as part of their routine care and thus are already at significant risk of blood

transfusions. Our research group has described a similar automated 24-hour sampling system for the measurement of tissue free cortisol in healthy adult volunteers (Bhake et al. 2013. The structure of this system has already been described in detail (R. C. Bhake et al. 2013). Briefly, the system is made from 3 parts (Figure 1): (1) microdialysis pump (CMA 107 pump, MDialysis, Sweden); (2) microdialysis catheter (66 linear microdialysis catheter, MDialysis, Sweden), (3) sample collector/fractioner (Designworks, Windsor, UK) (Figure 25). These components are interconnected by delicate tubing connectors that are assembled at the time of insertion. The original design has undergone several modifications to allow successful application in the operating theatre environment, where the catheter tubing is prone to kink or disconnect during surgery or patient transfer and as a result of clinical time pressures. Notably, the connections were reinforced by silicone tube wrapping, and most of the components are preassembled to reduce the insertion time.

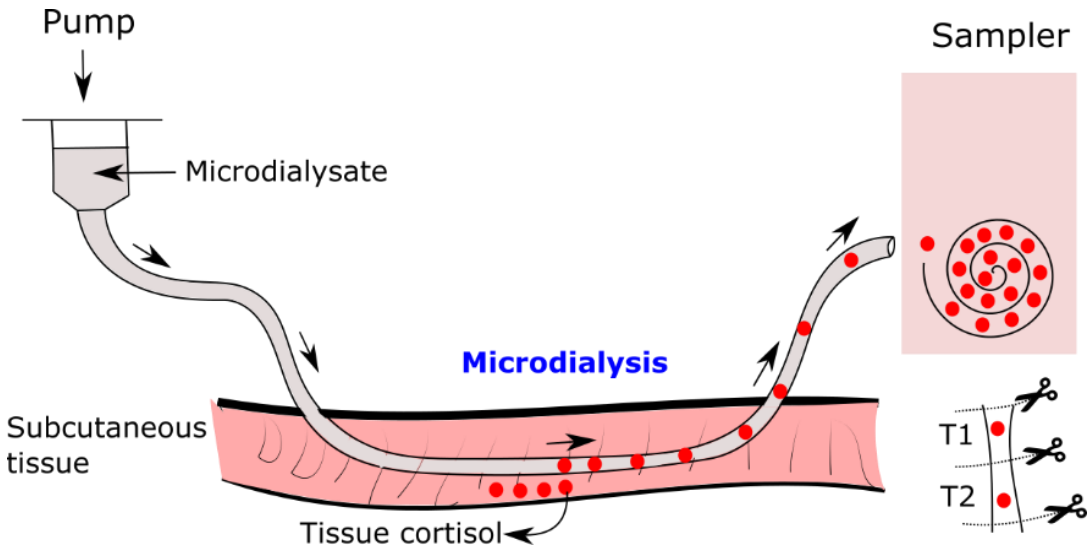

Figure 25 Overview of the microdialysis system

11.3.2 The pump

The microdialysis pump (CMA 107, Mdialysis, Sweden) (Figure 26) is set to perfuse fluid 1microL/min for 20-minute sampling. The fluid is pushed into the microdialysis catcher (MD 66 linear, Mdialysis, Sweden).

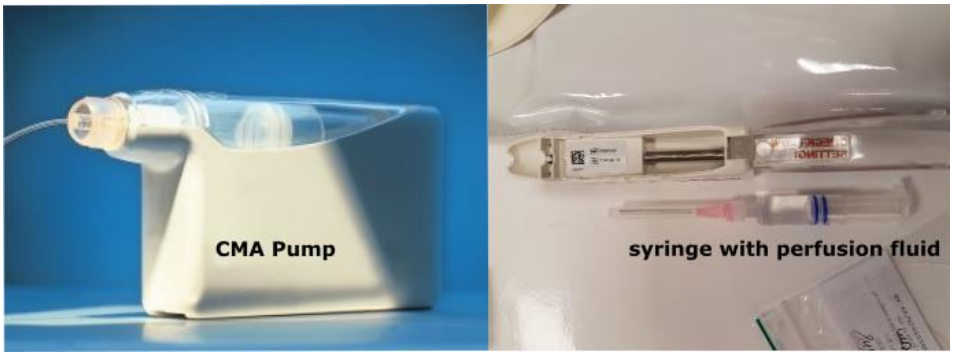

Figure 26 The Microdialysis pump

11.3.3 The microdialysis catheter

The microdialysis catheter has a 3-cm dialysis membrane that is placed subcutaneously via which the dialysis of cortisol in the perfusion fluid occurs. The dialysate is then pushed from the catheter, via FEP 20 cm cut tube into the sampling device (Figure 27).

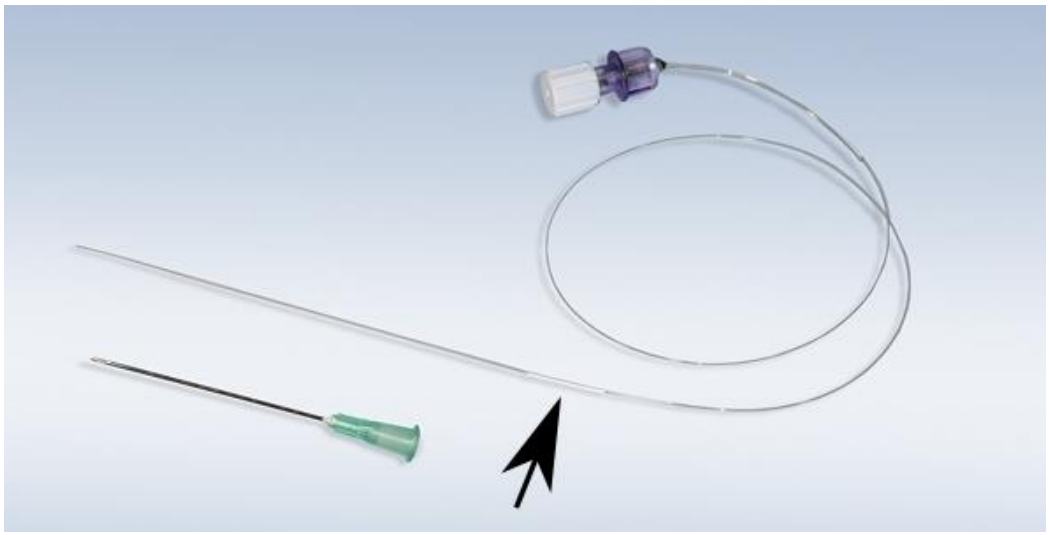

Figure 27 Microdialysis catheter (MD 66 type). The black arrow marks the membrane (Source: http://www.mdialysis.com/microdialysis-catheters/-66-linear-microdialysis-catheter)

11.3.4 The sampler

The sampler contains the sample store (e.g. the spool) and the mechanism that allows separation of the column of fluid that the sampler receives. This includes a pressure sensor, a servo pump, bubble pump and control electronics. A detailed description of the structure of the device can be found in Bhake et al. (R. C. Bhake et al. 2013) (Figure 28).

Figure 28 The Sampler with the spool (front lid removed)

11.3.5 The FEP tubing

The FEP (Fluorinated Ethylene Propylene) tubing is attached to the microdialysis catheter via so-called “pink-green” connector and the FEP tubing to the sampler device via a pink connector (Figure 29).

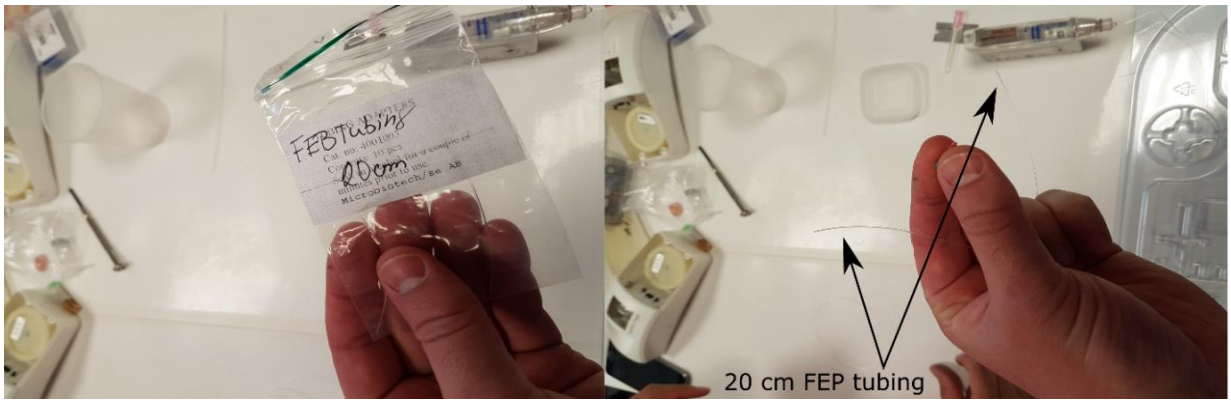

Figure 29 20-cm FEP tubing

11.3.6 The pink and the pink-green connectors

These set of connectors are pre-dilated in 100% alcohol prior to insertion into the tubing. The pink connector is used to secure the FEP tubing to the sampler (metal manifold) and the pink-green connector to secure the microdialysis tubing (Figure 30 and Figure 31).

Figure 30 "Pink-Green" connector

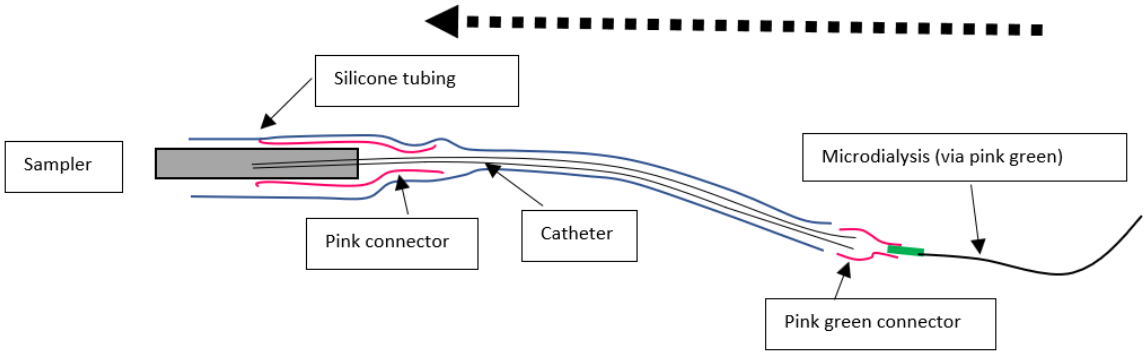

Figure 31 Figure to illustrate how the pink and pink-green connectors are used to assemble the microdialysis system. The dotted line represents the flow of fluid from the patients to the sampler.

11.3.7 The spool

We used the newest version of the sampling device that enables collection up to 30 hours without changing the spool. The device delivers air bubble every 20 minutes to separate each sample. The spool contains 3.8 m of tubing (Figure 32).

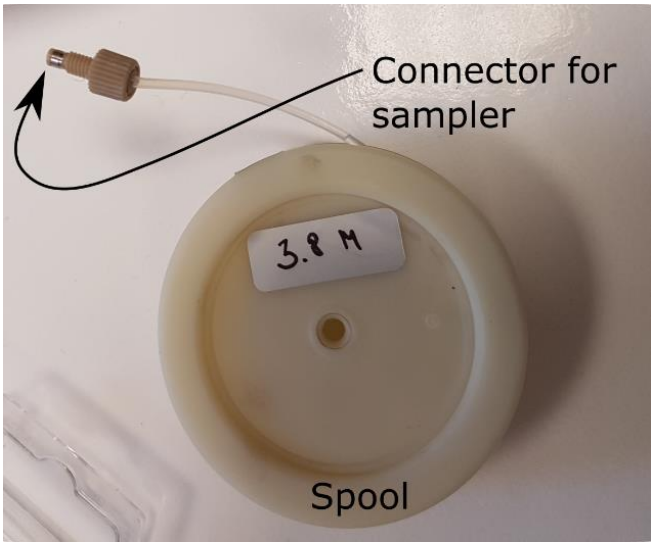

Figure 32 The spool.

11.3.8 Particularities of sampling of children undergoing heart surgery

Children undergoing heart surgery tend to be very mobile post-extubation (turning off sedation and removal of the breathing tube). Some patients will be extubated very early either on the operating table or in the intensive care unit (ICU) as part of enhanced recovery protocols. This practice increases the chances of the microdialysis tube disconnections. Moreover, during patient transfers, the tubing system is particularly vulnerable. To overcome this, our research group (Dr Thomas Upton) has designed silicone covers for the connectors. These are pre-dilated in petroleum-ether then applied to the connector. In contact with the air, these tubes shrink and strengthen the connections (Figure 33).

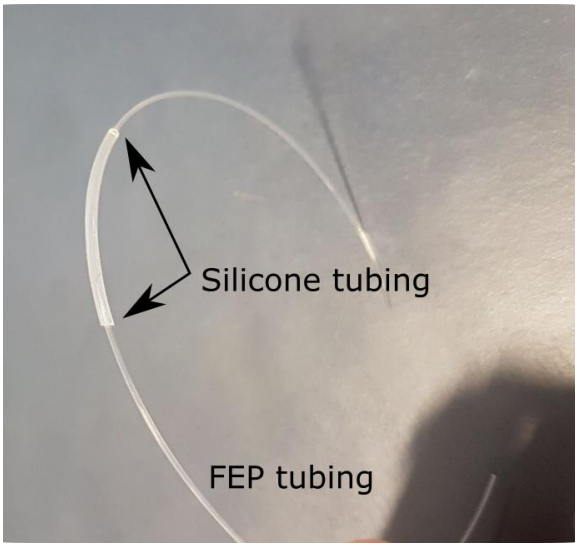

Figure 33 The Upton-Kershaw connector protector

11.3.9 The "FEP first" approach

Because of time pressure during theatre insertion, one essential modification of the technique was to pre-fit the FEP tube to the sampler. This minimises the time of connection of the FEP to the sampler but also time for the insertion of the silicon protector. This modification has resulted in an increase in the number of samples since its introduction. (Figure 34).

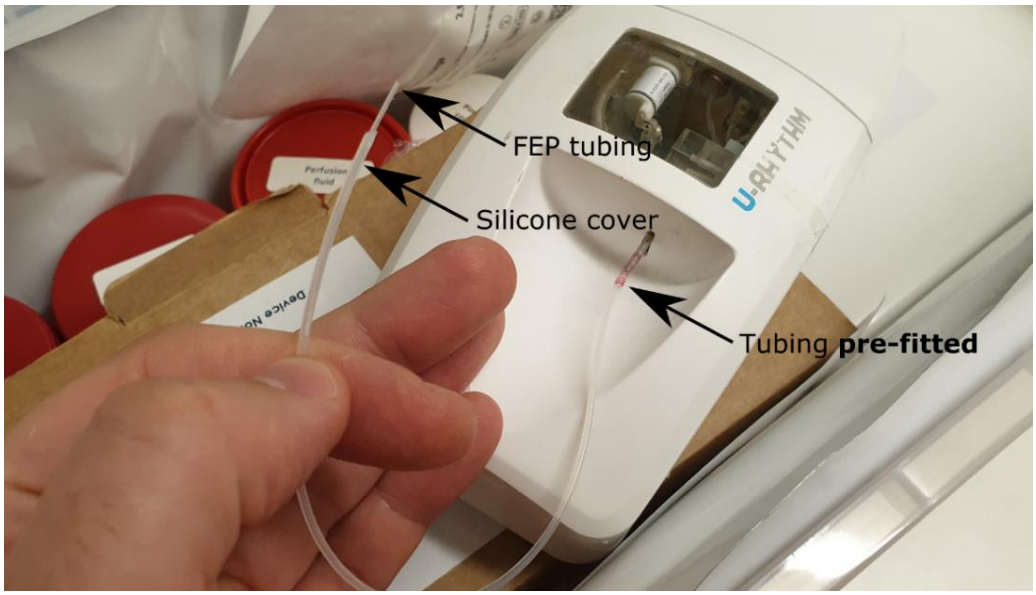

Figure 34 FEP – pre-fitted sampler

11.3.10 Insertion

Insertion was carried out after the patient has been anaesthetised, and all catheters (central or arterial) have been inserted. This was done under aseptic technique, in the operating theatre. Insertion was in the left lower abdominal wall. Compared to the studies on healthy volunteers (Upton et al., Ultradian Study, ultradian.blogs.bristol.ac.uk/), we did not dispose of the first samples. This is because we wanted to record cortisol pulsatility during the critical period of surgery (i.e. from knife to skin time). Figure 35 depicts the equipment necessary prior to insertion and Figure 36 the device *in situ*, during sampling.

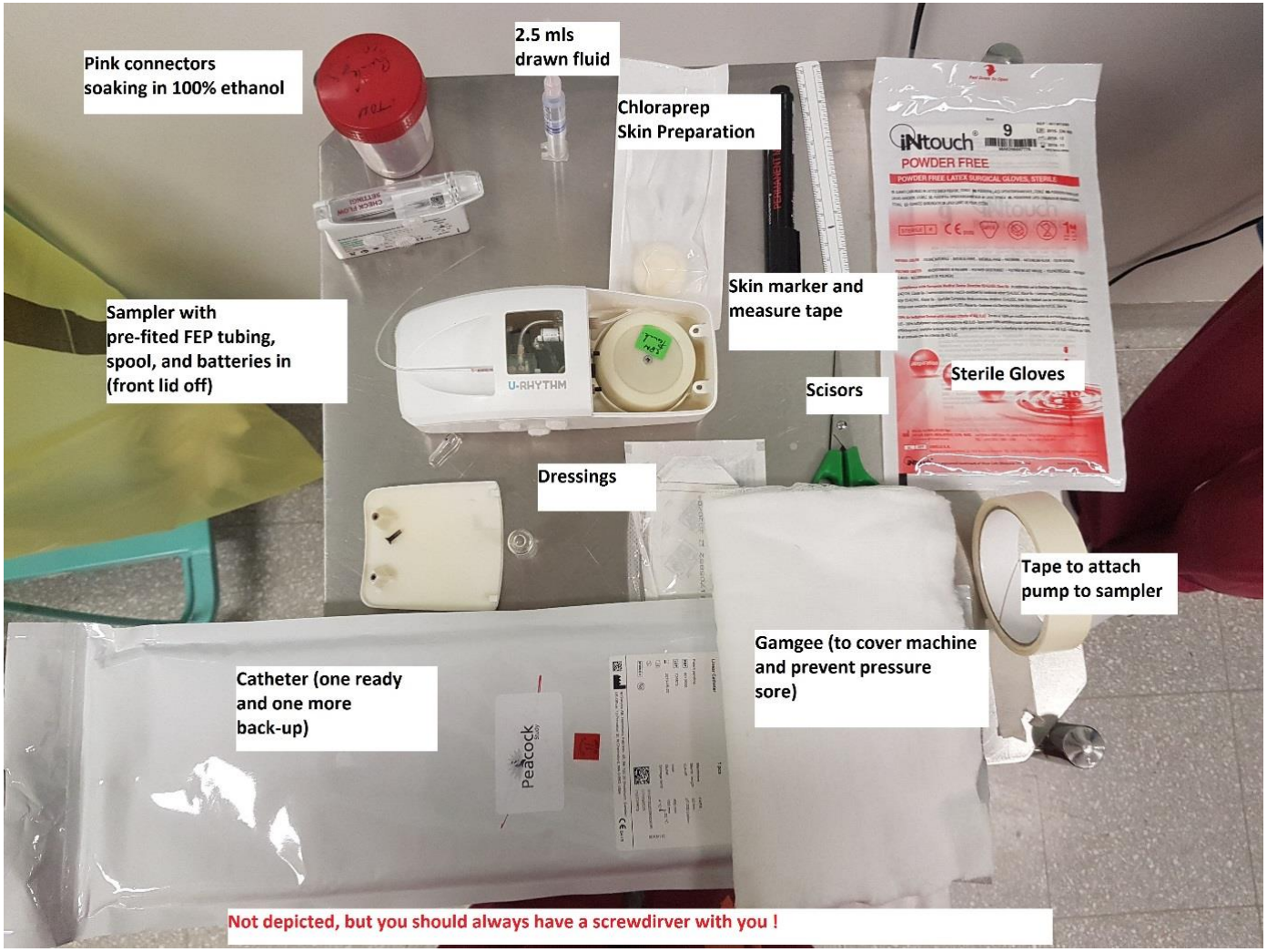

Figure 35 The setup of the table prior to insertion of the catheter

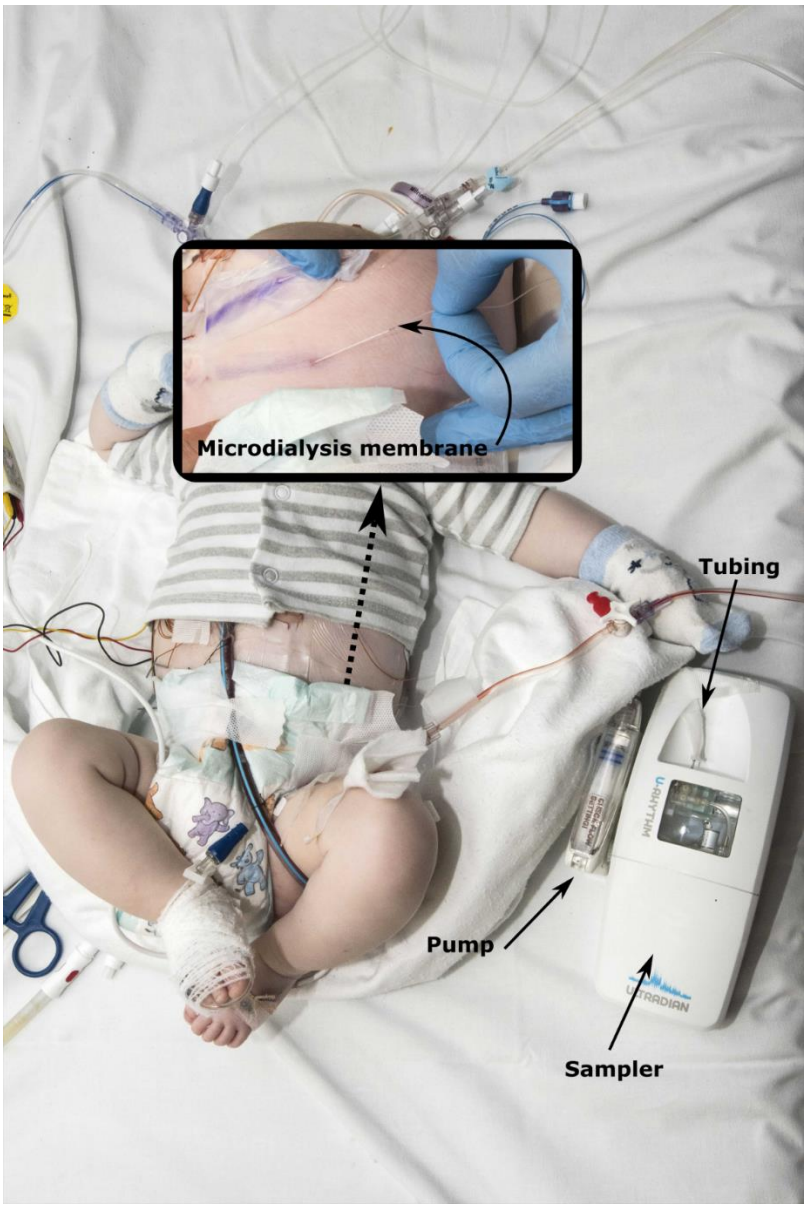

Figure 36 The microdialysis system in situ. The zoomed-in picture depicts the removal of the microdialysis membrane from the subcutaneous tissue

11.3.11 Disconnection

If the patient completed a 24-hour cycle of sampling, the catheter was disconnected according to the following sequence: (1) pump disconnection, (2) sampler disconnection and finally (3) removal of the subcutaneous microdialysis catheter.

11.3.12 Collection of samples

The spool was disconnected from the sampler after approximately 3 hours – this allowed for the pressures within the spool tubing to equalize. After the spool unwinds, this was secured on a metal plate using magnets (the Fudulu and Upton modification) to keep in place during sectioning of the samples (Figure 37). Each sample was marked, and photos were taken to be able to retrospectively analyse the quality of the spool/samples or re-interpret the time-points (Figure 38).

The samples were then cut and pushed in small Eppendorf tubes (Figure 38, 1) using an air syringe (Figure 38, 2), and then 10 microliters of the sample (Figure 38, 3) were pipetted in the 96 well plates (Figure 38,4). The cortisol levels were determined in each the sample using automated on-line solid-phase extraction-liquid chromatography-tandem mass spectrometry (SPE-LC–MS/MS) by our collaborators from the University of Groningen (see section 11.3.13).

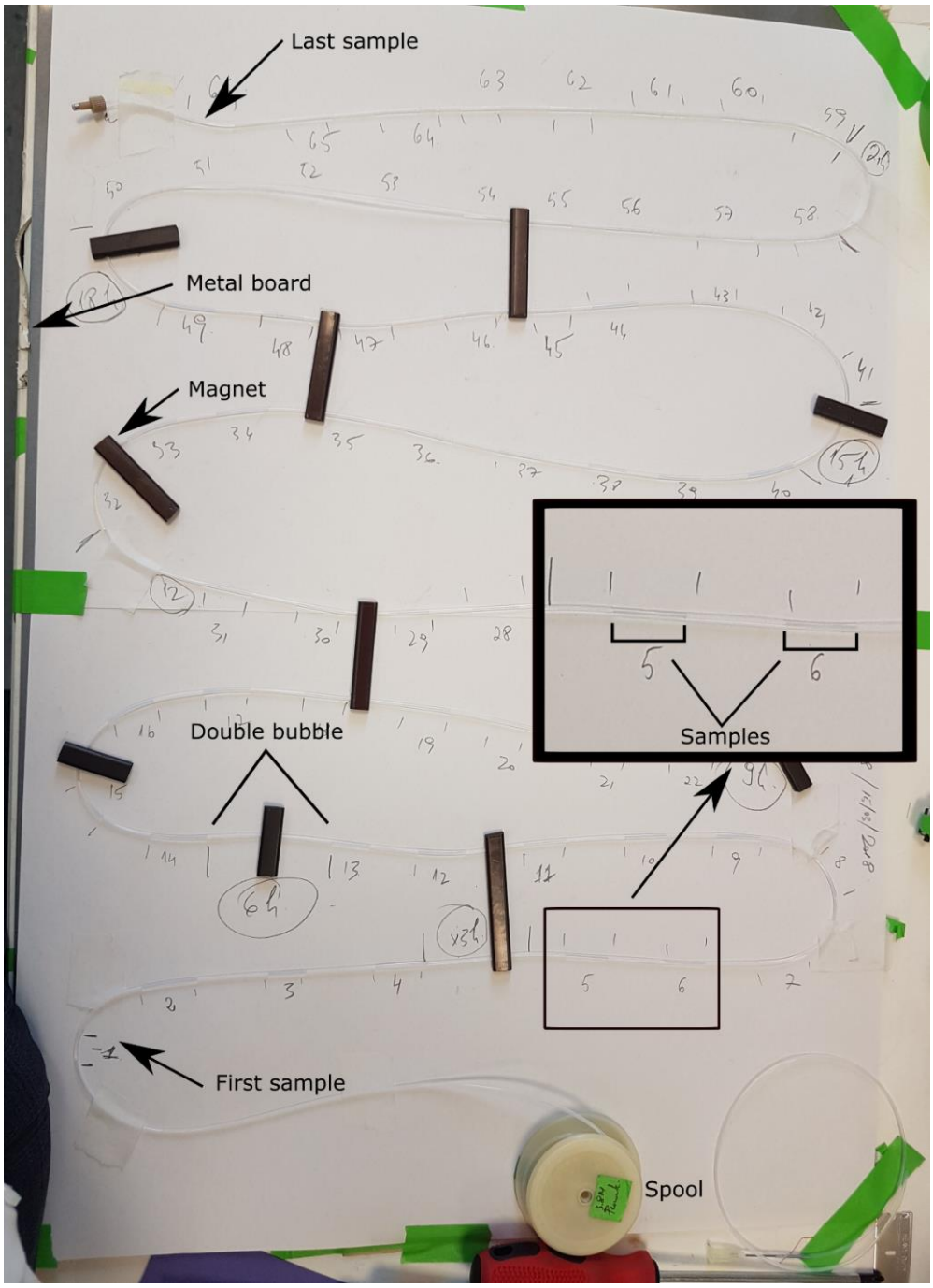

Figure 37 Twenty-four-hour sampling. The spool is opened and secured with magnets on a metal board.

11.3.13 Tissue cortisol and cortisone analysis

After the end of the 24-hour sampling period, the fluid samples (20 microliters/per sample), separated by air bubbles, were collected inside of a 4,5 m tubing that was rolled onto a spool. The spool was opened, and careful recording of the timing and separation of each sample was undertaken (Figure 38, 1). The samples were then pipetted in plates for further analysis.

The samples are isolated by cutting the tubing (between the air bubbles) (Figure 38,1). This can be a very tricky step because the plastic tubing has memory and acts as spring that throws the samples out. Therefore, we have implemented a metal board with magnets to stabilise the spool once this is unrolled. The samples are then pushed out of the sectioned tubing into small Eppendorf tubes using and air-filled syringe. From the Eppendorf containing 20 µL of the sample, half of this volume (10 µL) is pipetted into the 96-well plate (Waters) for further analysis. The samples are sent to Groningen, the Netherlands for analysis.

Cortisol and cortisone were measured in the microdialysate by isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) using cortisol-13C3 and cortisone-D7 as an internal standard. In brief, 10 µL dialysate was pipetted into a 96-well plate (Waters), 50 µL of internal standard and 90µL distilled water were added. The plate was mixed for ten minutes, and 2 µL was injected on the UPLC (Acquity) system equipped with a Kinetex Phenyl-Hexyl, 100 x 2.1 mm, 1.7 µm column (Phenomenex) in combination with a Xevo TQ-s with electrospray ionisation in selective reaction monitoring mode (Waters). For cortisol and cortisone, intra- and inter-assay variability were below 6% and 5%, respectively. Limit of quantification was 0.3 nmol/L for cortisol and 0.5 nmol/L for cortisone.

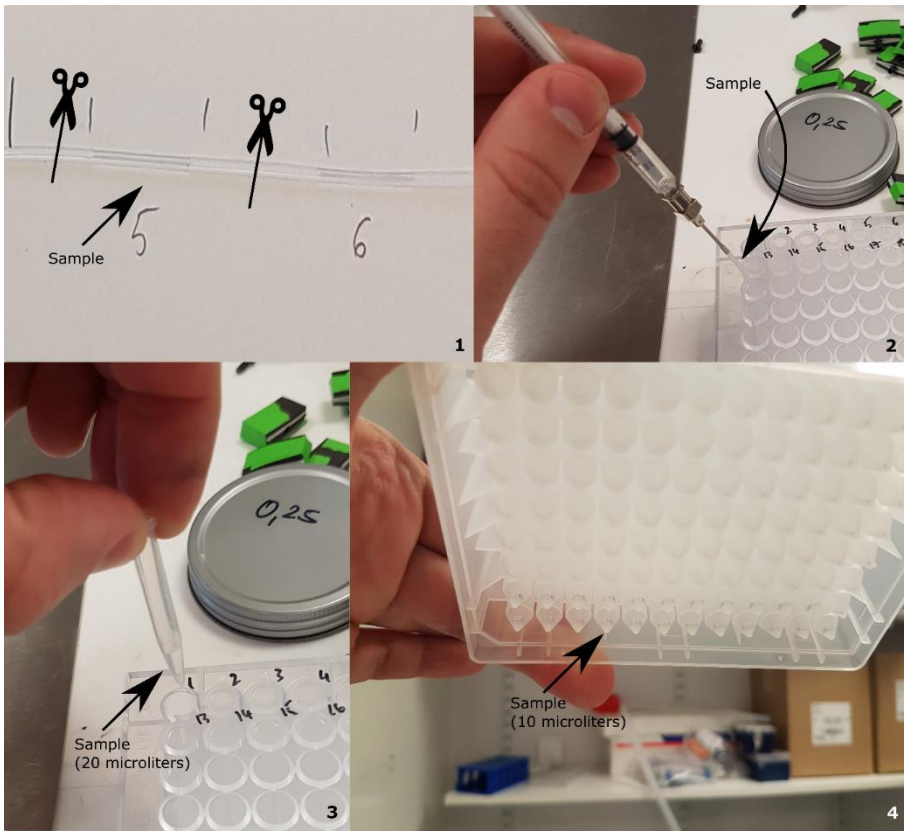

Figure 38 Preparation of samples for analysis

11.4 Study Protocol

11.4.1 Study design

11.4.1.1 The cardiac surgery cohort

This is a two-centre, descriptive study of the ultradian rhythms of cortisol that occur in neonates, infants and children. Forty-eight patients undergoing cardiac surgery will have 24-hour tissue cortisol/cortisone microdialysis sampling after induction of anaesthesia up to 24 hours (). Also, serum cortisol adrenocorticotrophic hormone (ACTH), interleukin 1 (IL-1), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 9 (IL8), interleukin-10 (IL-10) and tumour necrosis factor-alpha (TNF-α) will be measured at 7-time points for infants and older children. The time points are pre-op assessment clinic blood, anaesthesia induction sample, pre-CPB, post-CPB, 6 hours post anaesthesia induction, 12 hours post anaesthesia induction and 24 hours post anaesthesia induction (Figure 39). In neonates, due to circulating blood volume limits, we will measure the above variables at 6 time-points only (the post-CPB sample is excluded). Cortisol binding globulin (CBG) will be measured at 4 time points only: pre-op assessment clinic blood, anaesthesia induction sample, 6 hours post anaesthesia induction and 24 hours post anaesthesia induction. The cohort will be further split into 4 groups (Figure 40). Neonates (6 cyanotic and 6 acyanotic heart disease), infants (6 cyanotic and 6 acyanotic heart disease), 6 children aged 1-5 and, 18 patients aged 10-16. Acyanotic defects are classified based on pre-op oxygen saturation > 89% (on room air) while cyanotic heart defects based on a pre-op oxygen saturation ≤ 89% on room air.

For the 10-16 year's age group, we will recruit only the patients operated in the morning. This group will be further split into three subgroups. The pre-pubertal group (6 children) and the post-pubertal groups: 6 males and 6 females. Patients will be allocated to

either pubertal or post-pubertal group based on clinical criteria (e.g. presence of breast buds, periods, in girls and a testis volume greater than 4 mL in boys). The pre- and post-pubertal groups (18 patients) will have one morning sample of follicle-stimulating hormone (FSH), lutein hormone (LH) and oestradiol or testosterone.

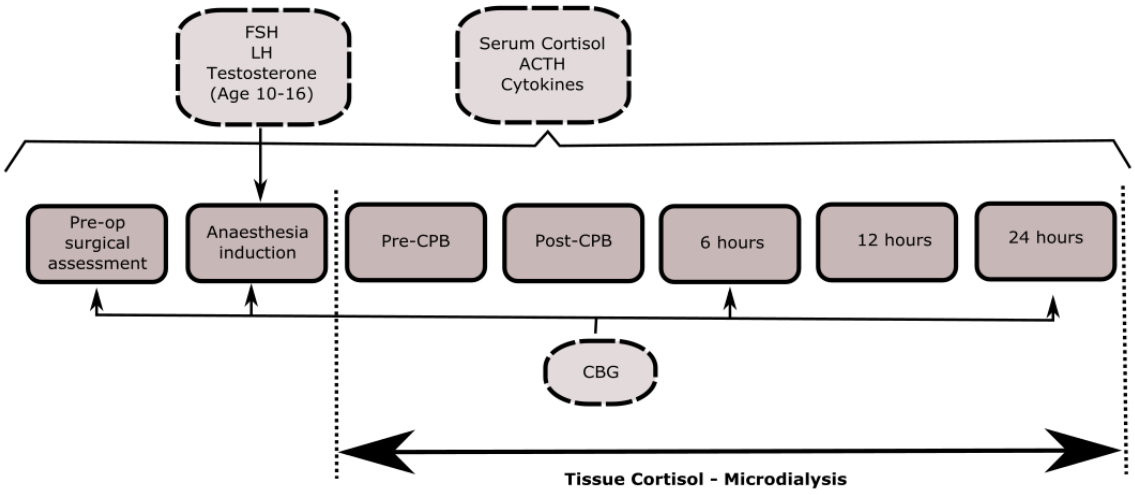

Figure 39 Sampling timepoints for the cardiac surgery cohort

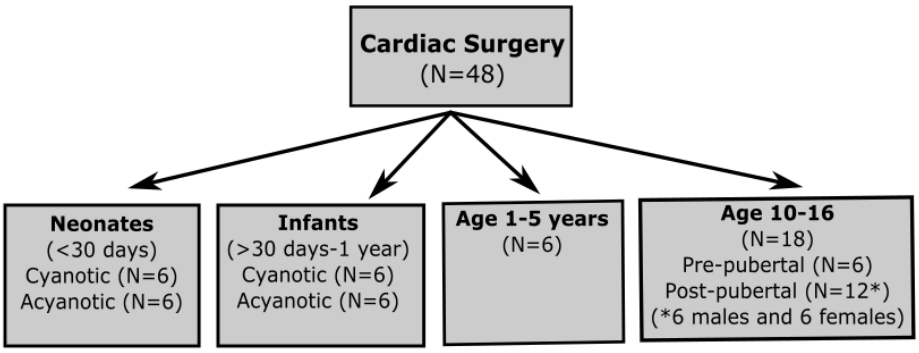

Figure 40 Recruitment groups for the cardiac surgery cohort

11.4.1.2 The cardiac investigation cohort

We will also recruit a cardiac investigation cohort of 30 children (no neonate group). In this group, we will recruit six infants and six children (1-5-year-old). We will also recruit 18 patients in the age group of 10-16 years. In this group, we will recruit six patients for the pre-pubertal group and 12 patients in the post-pubertal group (e.g. six males and six females) (Figure 41).

Similarly, to the cardiac surgery cohort, all patients have / will undergo continuous tissue microdialysis sampling for cortisol and cortisone after anaesthesia induction up to 24 hours, postoperatively. The cardiac investigation cohort patients will have only one basal pre-op sample via the cannula inserted for anaesthesia and microdialysis sampling for 24 hours or until the patient is discharged. From this sample, we will measure serum cortisol, CBG, ACTH and the same interleukins measured in the surgical groups (Figure 42).

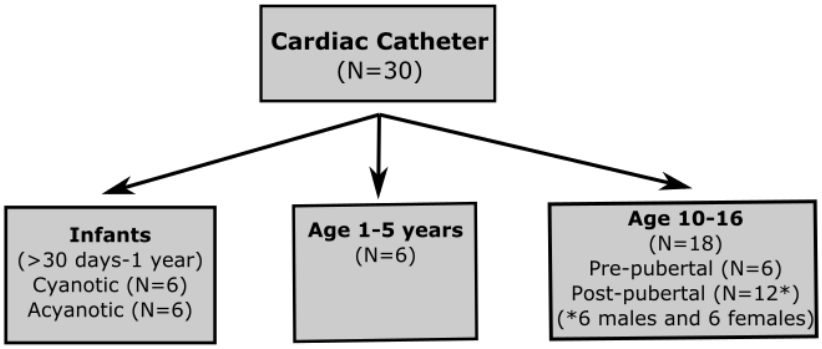

Figure 41Recruitment groups for the cardiac catheter cohort

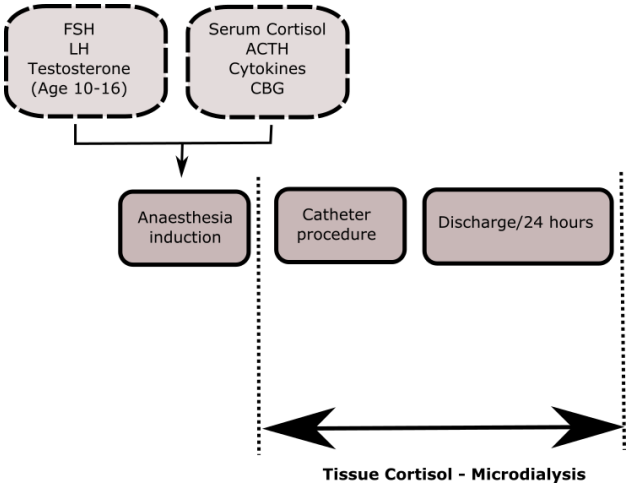

Figure 42 Sampling time points for the cardiac catheter cohort

11.4.2 Inclusion and exclusion criteria

11.4.2.1 Inclusion criteria for the cardiac surgery cohort

Participant may enter the study if ALL the following apply

1. Age 0-5 or 10-16 years
2. Undergoing cardiac surgery using CPB;
3. Weight above 2 kg for neonate patients.

We aim to recruit patients in each of the following categories:

- Neonates
- Infants
- Age 1-5
- Age 10-16

11.4.2.2 Exclusion criteria for the cardiac surgery cohort

Participant may not enter the study if ANY of the following apply:

1. Emergency operation

2. Current or recent (within 3 months) use of glucocorticoids
3. Disorders of the HPA axis
4. Thyroid disease

11.4.2.3 Inclusion criteria for the cardiac investigation cohort

Participant may enter the study if ALL of the following apply

1. Age 0-5 or 10-16 years
2. Undergoing minimally invasive cardiac investigation with anaesthesia.
3. Weight above 2 Kg.

We aim to recruit patients in each of the following categories:

- Infants
- Age 1-5
- Age 10-16

11.4.2.4 Exclusion criteria for the cardiac investigation cohort

Participant may not enter the study if ANY of the following apply:

1. Emergency investigation
2. Current or recent (within 3 months) use of glucocorticoids
3. Disorders of the HPA axis
4. Thyroid disease

11.4.3 Primary and secondary outcomes

11.4.3.1 Primary outcome

The primary outcome is the subcutaneous cortisol profile during the 24-hour measurement period for both cohorts.

- Tissue cortisol levels in all subjects are measured using subcutaneous microdialysis. For the surgical cohort, total serum cortisol will also be measured at 7 time points (pre-op assessment, pre-operative – before anaesthesia induction, pre CPB, post CPB, at 6, 12 and 24 hours postop) for infants and pre- and post-pubertal children and at 6 time points for neonates (post-CPB sample excluded). These will be correlated with tissue cortisol levels obtained by microdialysis over 24 hours.
- For the cardiac investigation cohort, serum cortisol will be measured pre-procedure (1-time point) for all age groups.
- Pulsatility and interaction of cortisol and ACTH as assessed using a bespoke algorithm developed by our group.

11.4.3.2 Secondary outcomes

For the surgical cohort:

- Adreno-corticotropic hormone (ACTH) Measured at seven-time points (pre-op assessment, pre-operative, pre CPB, post-CPB, at 6, 12 and 24 hours postop) for infants and pre- and post-pubertal children and at six-time points for neonates (post-CPB sample excluded).
- Cortisol Binding Globulin (CBG) measured at four-time points (pre-op assessment, induction, at 6, and 24 hours postop) for. all age groups.
- IL-1, IL-4, IL-6, IL8, IL10 and TNF-alpha measured at seven time-points (pre-op assessment, pre-operative, pre CPB, post-CPB, at 6, 12 and 24 hours postop) for infants and pre- and post-pubertal children at and six-time points for neonates (post-CPB sample excluded).
- FSH, LH and testosterone or oestradiol for 10-16-year olds only (pre-operative – before anaesthesia induction)
- Measure the following clinical outcomes to correlate with cortisol rhythms:

a. Death;

b. Preoperative biventricular function;

c. Cardiac arrest;

d. Extracorporeal membrane oxygenation use;

e. Renal insufficiency (creatinine more than two times normal);

f. Hepatic insufficiency;

g. Duration of mechanical ventilation post-cardiac surgery;

h. Inotrope and vasopressors use

i. Intensive care unit stay;

j. Hospital Stay;

k. Infection;

l. Insulin use;

m. Fluid retention (daily weights)

For the cardiac investigation cohort:

- Adreno-corticotropic hormone (ACTH) measured at 1 timepoint (pre-operative)
- Cortisol Binding Globulin (CBG) measured at 1 timepoint (pre-operative)
- . IL-1, IL-4, IL-6, IL8, IL10 and TNF-alpha measured at 1 timepoint (pre-operative)
- FSH, LH and testosterone or oestradiol for 10-16-year olds only (pre-operative - before anaesthesia induction)
- Measure the following clinical outcomes to correlate with cortisol rhythms:

a. Death;

b. Preoperative biventricular function;

c. Cardiac arrest;

d. Renal insufficiency (creatinine more than two times normal);

e. Hepatic insufficiency;

f. Hospital Stay;

g. Infection;

h. Insulin use.

11.4.4 Sample size calculation

The lack of previous studies does not allow power calculation on the expected variations in ultradian rhythmicity of the HPA axis in children. However, the use of multiple samples from the microdialysis provides high discriminatory power and recent investigations from our group demonstrated very marked changes in pulse characteristics in a group of 20 adult patients undergoing CABG compared to healthy individuals (Ben Gibbison et al. 2015). Furthermore, a study of 10 patients with obstructive sleep apnoea pre- and post- continuous positive airway pressure demonstrated significant changes in ACTH/cortisol ultradian patterns (David E Henley et al. 2009).

11.4.5 Research procedures

The **cardiac surgery cohort** will have blood sampling for serum cortisol, ACTH, CBG. IL-1, IL-4, IL-6, IL-8, IL10 and TNF-alpha.

To minimise patient distress, we will synchronise a *preoperative*, basal, sample in the preoperative assessment clinic and before anaesthesia *induction*, along with other routine preoperative blood samples.

Intraoperatively, samples will be collected from the arterial or central line, *pre-CPB* and *post CPB.*

Postoperatively, blood samples will be spaced at 6, 12 and 24 hours postoperatively.

Infants, pre- and post-pubertal children: At each time point, we will measure serum cortisol, ACTH and interleukins. CBG will be measured at 3-time points over 24 hours. (induction, 6 hours and 24 hours only). This results in a total blood volume of 1.3mL per draw for time points, including CBG sampling and 1.0ml for time points without CBG. If we measure at six-time points within 24 hours (e.g. we exclude the pre-assessment clinic sample), this results in a cumulative, perioperative blood volume draw of 6.9 mL. Previous policies and recommendations reported the safe limits of blood sampling for research in healthy children and the proposed study is well within limits (Howie 2011). Most guidelines quote a maximum cumulative draw volume ranging from 5-10 % of the total circulating volume, 50 mL total over 8 weeks (whichever is less) and the lowest volume quoted is of 3mL/kg in critically ill children.

Neonates: Neonates with cardiac disease are by their nature smaller than children without cardiac disease and therefore constitute a particular case. Hazinski et al. (Hazinski 2012) reported the circulating volumes at various ages from which we can calculate the circulating volume depending on weight, and calculate the maximum 5 % cumulative blood volume. For example, the circulating volume of a 2 kg neonate is 180 mL (90mL kg x 2), and 5 % of this volume is 9 mL. However, to be within all guidelines, we will use the 3mLs/kg limit. This would result in 3 x 2=6 mL maximum blood volume for measurements. This means that we would have to limit the weight in neonates to 2 kg and reduce the number of sampling time-points within the 24 hours from 6 to 5. In this case, we will omit the post CPB sample (see Figure 7) in order to be within the safety limits of all guidelines. This results in a cumulative blood volume for 24 hours of 5.9 mL, which is below the 6 mL lowest limit of the guideline.

For the **cardiac investigation cohort**, blood sampling for serum cortisol, ACTH, CBG and IL-1, IL-4, IL-6, IL8, IL10 and TNF-alpha will take place at only one-time point pre-

operatively. For this group, the weight limit can be lowered because of the need for only 1 sample (1.3 mL total draw). Therefore, blood sampling will be theoretically possible in the cardiac cohort in neonates with a weight above 0.33 kg (although few, if any babies are likely to be as small as this).

Modified ultrafiltration with CPB is commonly used in paediatric cardiac surgery to haemoconcentrate patients but also to reduce the systemic inflammatory response to CPB. Several studies demonstrated a decrease in the levels of specific pro-inflammatory cytokines such as IL6, IL8 or TNFα (Ziyaeifard, Alizadehasl, and Massoumi 2014). Although this could alter the levels of IL6 that will be measured in the blood, we believe that the free cortisol measured by subcutaneous microdialysis will not be affected because of the lipophilic nature of its molecule and a large volume of distribution.

It is common for paediatric surgery patients to receive blood products such as red blood cell packs, fresh frozen plasma or Cryoprecipitate perioperatively. We were also planning to assay cortisol and ACTH in 10 units each of fresh frozen plasma or Cryoprecipitate, to determine the level of hormones in these blood products and any impact it may have on our analyses. However, we have not done after a discussion with NHS Blood and Transplant that advised us there is no cortisol in these blood products.

11.4.6 Serum cortisol analysis

The serum cortisol samples were analysed by the University of Bristol NHS Foundation Trust, Department of Biochemistry.

11.4.6.1 Principle and Method of the Procedure Used for the Examination

We used the Elecsys Cortisol II assay that makes use of a competition test principle using a monoclonal antibody which is directed explicitly against cortisol. Endogenous cortisol which has been liberated from binding proteins with danazol competes with exogenous cortisol derivative in the test which has been labelled with ruthenium complex for the binding sites on the biotinylated antibody (Morgan 2019).

11.4.6.2 Test principle

Competition principle. The total duration of the assay: 18 minutes (Morgan 2019).

- 1st incubation: 10 µL of sample is incubated with a cortisol-specific biotinylated antibody, and a ruthenium complex labelled cortisol derivative. Depending on the concentration of the analyte in the sample and the formation of the respective immune complex, the labelled antibody binding site is occupied in part with sample analyte and in part with ruthenylated hapten.
- 2nd incubation: After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

- Results are determined via a calibration curve which is an instrument specifically generated by 2-point calibration and a master curve provided via the reagent barcode or e-barcode.

11.4.6.3 Reportable Intervals

- 3-1750 nmol/L (defined by the lower detection limit and the maximum of the master curve) (Morgan 2019).

11.4.6.4 Results outside Reportable Intervals

Values below the lower detection limit are reported as <3.0 nmol/L. Values above the measuring range are reported as >1750 nmol/L (Morgan 2019).

11.4.7 Serum ACTH analysis

The serum ACTH samples were analysed by the University of Bristol NHS Foundation Trust, Department of Biochemistry.

11.4.7.1 Principle and Method of the Procedure Used for the Examination

All blood ACTH samples were handled on ice. The Elecsys ACTH assay was used. This assay employs two monoclonal antibodies specific for ACTH and for the C-terminal region. Due to common antigenic structure, the antibodies recognise intact biologically active ACTH and the ACTH precursors: pro-opiomelanocortin (POMC) and pro-ACTH (Morgan. 2019).

11.4.7.2 Test principle

Sandwich principle: Total duration of assay=18 minutes (Morgan. 2019).

- 1st incubation: 50µL of the sample, a biotinylated monoclonal ACTH-specific antibody, and a monoclonal ACTH-specific antibody labelled with a ruthenium complex react to form a sandwich complex.

- 2nd incubation: after addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.

The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

11.4.7.3 Limits and ranges

Measuring range:

- 1.0-2000 ng/L (defined by the lower detection limit and the maximum of the master curve). Values below the lower detection limit are reported as < 1.00 ng/L or. Values above the measuring range are reported as > 2000 ng/L.

The lower detection limit is 1.00 ng/L. The lower detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying two standard deviations above that of the lowest standard (master calibrator, standard 1 + 2 SD, repeatability study, n = 21) (Morgan. 2019).

11.4.8 Serum and tissue cortisol: levels to expect and correlations between blood and tissue compartments

One pertinent question for the Peacock study is what levels of serum and tissue cortisol measurement we would expect. There is no study to date in children that investigated the correlation between blood cortisol and tissue cortisol. However, Cohen et al. (Cohen et al. 2009), in a prospective observational study of 10 critically ill patients with burns, measured the

plasma cortisol (total and free) in blood and the interstitial (tissue) cortisol using microdialysis in the burn and non-burn tissue. The authors used a Cortisol analysis was by ELISA for cortisol analysis. The mean total plasma cortisol was 8.8 +/- 3.9 mcg/ dL (242.7 +/- 107.6 nmol/L) and mean free plasma cortisol was 1.7 +/- 1.1 mcg/ dL (46.9 +/- 30.3 nmol/L). The corresponding tissue cortisol level measured by microdialysis was 0.80 +/- 0.31 mcg/dL (22 +/- 8.5 nmol/L) in the burn areas and 0.74 +/- 0.41 mcg/dL (20.4 +/- 11.3 nmol/L) in the non-burn areas with no significant difference between burn and non-area p=0.8). In a recent study by Bhake et al. (R. Bhake et al. 2019) on healthy volunteers correlated the total and free blood cortisol with tissue microdialysis cortisol after 250 µg Synacthen or 1 mg dexamethasone. They measured simultaneously cortisol in blood and tissue, every 10 minutes using automated devices. There was a good correlation between tissue and blood cortisol with no significant delay (e.g. less than 5 minutes). For example, in one of their experiments, the median serum total cortisol pre-Synacthen was 321 nmol/l (range 188-773) and at the peak was 973 nmol/l (range 778-1369) between 2 and 3 hours post-Synacthen. The corresponding median subcutaneous tissue cortisol levels obtained by microdialysis were 11.5 nmol/l (range 2.3-38.5) pre-Synacthen and at peak 67.4 nmol/l (18.7-101.5) between 1-3.25 hours post-Synacthen.

11.4.9 CBG assays

The CBG was analysed using a commercially available kit (Kip1809) DiaSource Louvain-La-Neuve, Belgium.

Method

A fixed amount of 125I labelled CBG competes with the CBG present in the sample or in the calibrator for a fixed amount of anti-CBG antibody sites, which are bound to the goat anti-mouse antibodies immobilised to the wall of a polystyrene tube. After 2 hours incubation at

room temperature, an aspiration step terminates the completion reaction. The tubes are then washed with 2ml of working wash solution and aspirated again. A calibration curve is plotted, and the CBG concentrations of the samples are determined by dose interpolation from the calibration curve (DIAsource ImmunoAssays 2019).

Reagents

- Calibrators: Reconstitute the zero calibrators with 3 ml distilled water and the other calibrators with 1 ml distilled water.
- Controls: Reconstitute the controls with 0.5 ml distilled water.
- Working Wash solution: Prepare an adequate volume of Working Wash solution by adding 69 volumes of distilled water to 1 volume of Wash Solution (70x) (2ml+138ml, 3ml+207ml). Use a magnetic stirrer to homogenise. Discard unused Working Wash solution at the end of the day.

Specimen collections and preparation

- Serum samples were kept at 2-8°C.
- If the test is not run within 48 hrs, storage at -20°C was recommended.
- Freeze-thaw cycles should be and were avoided
- After thawing, the samples should be mixed and centrifuged.
- The samples must be diluted 25 times in Dilution Buffer.
- Recommended procedure: 100 µl serum + 2.4 ml Dilution Buffer. Use LP4 tubes.

Procedure

1. Label coated tubes in DUPLICATE for each calibrator, control and sample. For the determination of total counts, label 2 standard tubes.

2. Briefly vortex calibrators, controls and diluted samples and dispense 100µl of each into the respective tubes.

3. Dispense 100 µl of 125Iodine labelled CBG into each tube, including the tubes for total counts.

4. Dispense 100 µl of CBG antiserum into each tube (except total counts).

5. Shake the tube rack gently by hand to liberate any trapped air bubbles.

6. Incubate for 2 hours at room temperature with continuous shaking at 300 rpm.

7. Aspirate (or decant) the content of each tube (except total counts). Be sure that the plastic tip of the aspirator reaches the bottom of the coated tube in order to remove all the liquid.

8. Wash tubes with 2 ml Working Wash solution (except total counts) and aspirate. Avoid foaming during the addition of the Working Wash solution.

9. Let the tubes stand upright for two minutes and aspirate the remaining drop of liquid.

10. Count tubes in a gamma counter for 60 seconds.

Calculation of Results

The concentrations read on the calibration curve for the samples and controls must be multiplied by 25 (dilution factor) (DIAsource ImmunoAssays 2019).

11.4.10 Cytokine analysis

Cytokine analysis was performed in duplicate using MILLIPLEX® MAP multiplex kits on Luminex MAGPIX® system. The assay uses a mix of fluorescent-coded magnetic beads, each set coated with a specific capture antibody. Corresponding analytes (IL-1β, IL-4, IL-6, IL-8, IL-10 and TNFα) are bound to the bead during an overnight incubation with patient serum samples taken at various time points. A biotinylated detection antibody is then added, before

incubation with Streptavidin-PE conjugate reporter molecule. Each bead is identified, and a fluorescent reporter signal measured by the MAGPIX® system.

11.4.11 Participant recruitment

Patients undergoing non-emergency heart surgery and cardiology catheter were and will be invited to participate.

11.4.11.1 Consent +/- assent will be obtained according to patient age:

- 0-5 years: Parents/guardians of patients are expected to read and understand the parent/guardian information leaflet and (with the assistance of a member of the clinical team) explain the purposes and consequences of the study to the patient at a level suitable for their age, if appropriate. Parents/guardians are required to provide full, written, informed consent.

- 10-15 years: Patients who can read and understand will be provided with a PIL, and parents/guardians will be provided with a parent/guardian information leaflet. Parental consent must be obtained for enrolment to be valid. In parallel, where appropriate, the patients will be given a suitable form to provide written informed assent to participate in the study. Failure to complete and sign this assent form will be considered as a refusal to participate. This refusal should be respected.

- 16 years: Patients aged 16 years are legally responsible for providing their own consent and will be provided with their own information leaflet and consent form. Parental signed consent can be taken besides using the parent/guardian consent form; however; this is not a necessity and alone is not sufficient for enrolment.

Patients can be formally enrolled, and consent/assent obtained at various points preoperatively.

Details of all patients approached for the study and reason(s) for non-participation (e.g. reason for being ineligible or patient refusal) were documented.

11.4.11.2 Patients waiting at home

During the first outpatient clinic, the parents/guardians of patients considered eligible for the study will be provided with an information leaflet. Following their meeting with the consultant surgeon, a member of the research team explained the details of the study in person and provided answers to any questions that might be raised. At this time, patients were also provided with a Patient Information Leaflet (PIL) suitable for their age group. Approaching parents/guardians and patients during their first clinic, ensured maximum time is given to consider participation. All information leaflets were approved by the Research Ethics Committee (REC).

Consent/assent will be requested at the pre-assessment clinic (1-2 weeks prior to procedure). If consent/assent has not been sought before the patient's arrival at the hospital for surgery, parents/guardians/patients have the opportunity to provide signed consent/assent up to 4 hours prior to the scheduled surgery time.

On the rare occasions where parents/guardians/patients cannot be seen during pre-operative clinics, the procedure for patients waiting in-hospital will be followed.

Information leaflets may occasionally be sent directly to the patient's home address with an invitation letter if a patient does not attend the outpatient clinic, or if they cannot be approached during the clinic visit.

11.4.11.3 Patients waiting in-hospital

The parents/guardians of patients considered eligible for the study will be given a parent/guardian information leaflet when the patient is transferred to a ward at the hospital. The patients will also be given a PIL suitable to their age group at this time.

Admission/transfer to the hospital is usually the day before their procedure. Parents/guardians and patients should, therefore, have a minimum of 24 hours to read and consider the information and to ask questions before being asked to decide whether they are willing to consent/assent.

On occasions, this time interval may be as little as (but never less than) 4 hours, for example for patients admitted for same-day surgery without prior notification. It is essential to include these patients for the applicability of the study findings.

11.4.12 Ethical considerations

11.4.12.1 Review by an NHS Research Ethics Committee

Ethical approval was obtained from a UK National Research Ethics Service (NRES) Research Ethics Committee, South West – Frenchay Research Committee, REC reference: 11/H0107/9.

Any amendments to these documents, after a favourable opinion from the REC has been given, were submitted to the REC for approval before implementation.

11.4.13 Risks and anticipated benefits

11.4.13.1 Potential benefits to participants:

There are no direct benefits to participants.

11.4.13.2 Potential harms to participants:

The only anticipated harms to participants would be from taking blood samples and potential risk for superficial infection associated with the microdialysis catheter. This volume of blood taken, however, is small and would not make the difference between needing and not needing a blood transfusion. A critical care patient in the intensive care unit would expect up to half this amount of blood taken per day for clinical blood tests.

The potential risk of infection at the site of subcutaneous catheter insertion is extremely low, and we shall minimise this even further by adherence to a rigorous aseptic, non-touch technique and keeping the catheter in for only 24 hours.

Over the last 6 years during which we have undertaken many studies both inpatients and normal volunteers, we had no infections related to subcutaneous catheter insertion. In

keeping with this a study by Poca et al. (M.A. et al. 2006) on 97 neurocritical adult patients who underwent percutaneous brain microdialysis catheter insertion, reported a 0% infection rate. Furthermore, in a literature review by Hack et al. (Hack et al. 2005) of subcutaneous microdialysis in paediatric patients, including neonates with extremely low birth weight, no infections were reported.

Despite this very low risk, we will regularly monitor the insertion site for any signs of infection throughout catheter insertion.

11.4.13.3 Possible adverse effects of each intervention:

The risk of taking the blood samples at 7 time points from existing *in situ* lines is virtually nil.

The risk of superficial infection at the site of subcutaneous microdialysis catheter insertion.

11.5 Statistical analysis

Graph Pad Prism version 7.00 (Graph Pad Software, La Jolla, CA, USA) was used for statistical analysis and data graphing. For the grouped analyses, all data are expressed as mean ± SEM. Calculation of the AUC for cortisol and per hour was done using a specific formula calculation in Microsoft Office Excel 365 (e.g. the sum of the area of all trapezoids that can be drawn beneath the curve) (Microsoft Corporation, Redmond, Washington, United States).

If values on the x-axis are inputted in column A starting from row 1 and the values corresponding to the y-axis are inputted in column B the formula input in column C was C1 "=(B1+B2)/2*(A2-A1)". The area under the curve between certain time points was deducted by calculating the sum of totals in that interval.

Total AUC for Cortisol was crosschecked with AUC calculation done in Graph Pad prism. Since the distribution of the AUC for cortisol within the groups was skewed, we used nonparametric tests for between-group comparisons (Mann Whitney test or one-way ANOVA test followed by Kruskal-Wallis test for the post hoc analysis). The preliminary analysis of CBG and cytokine data was done using a two-way ANOVA test followed by a Tuckey's multiple comparisons test. Statistical significance was set for all statistical tests at $P<0.05$.

11.6 Results

11.6.1 Overview of recruitment

The study started recruitment in 14/09/2017. To date, a total of 69 children (61/78, 84 % of recruitment target) consented to take part in the study (49 surgical and 20 catheters). Bristol Children's Hospital recruited a total of 53 patients (53 surgical and 17 catheters) while 16 patients (15 from the surgical group and one from the catheter group) consented to take part in the study at Royal Brompton Hospital, London, UK. Out of 69 children that underwent sampling, we obtained complete data for 45 patients (45/78, 64%% of the original recruitment target). The rest of the sampled patients (N=24) were withdrawn due to various reasons: (1) accidental early disconnection (below threshold of 8 hours) (N=13); (2) system failure (N=4), (3) administration of glucocorticoids during sampling (N=3), (3) sampling), (4) no surgeon to insert the catheter (N=2) and (5) damage during transport of samples from London to Bristol (N=2) . Herein, the preliminary analysis was run on 36 patients for which data was available.

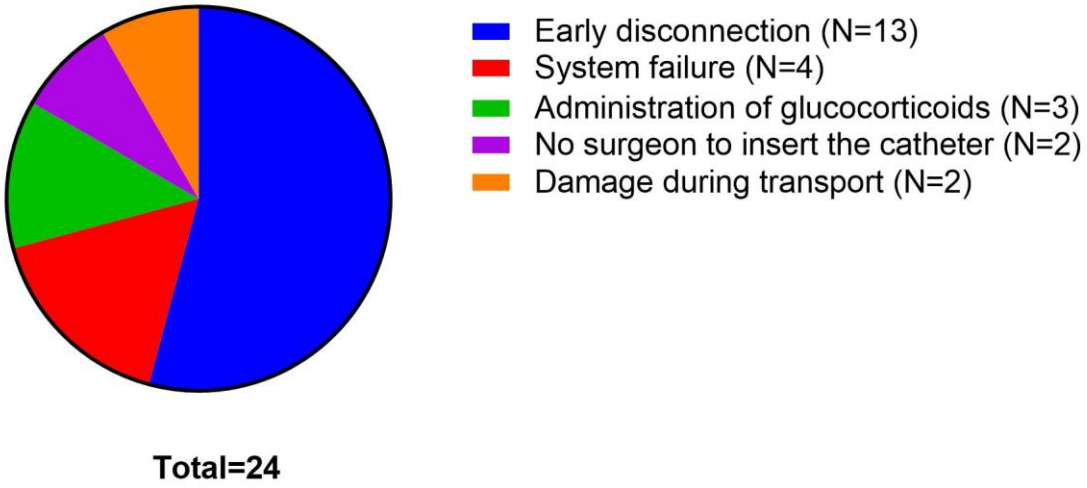

Figure 43 Withdrawal causes

11.6.2 Preoperative patient characteristics

Patient	Age	Weight (kg)	Group	Surgical procedure	RACHS Risk Category*	Preoperative oxygen saturation (%)
P1	11 days	3.5	<30 days	Transposition of great arteries repair	4	94
P2	12 days	3.5	<30 days	Transposition of great arteries repair	3	88
P3	13 days	5	<30 days	Transposition of great arteries repair	3	78
P4	12 days	3	<30 days	Right ventricle to pulmonary artery conduit	3	87
P5	8 days	3.1	<30 days	Interrupted aortic arch repair	3	89
P6	3 months	4.5	30 days -1 year	Ventricular septal defect repair	2	92
P7	5 months	6	30 days -1 year	Tetralogy of Fallot Repair	3	92
P8	3 months	6.6	30 days -1 year	Aortic valve repair	3	98
P9	5 months	6	30 days -1 year	Left atrioventricular valve repair	3	100
P10	4 months	5.1	30 days -1 year	Tetralogy of Fallot Repair	3	79
P11	4 months	5.1	30 days -1 year	Glenn shunt and diaphragm plication	2	76
P12	7 months	5.3	30 days -1 year	Glenn shunt	2	86
P13	6 months	4.9	30 days -1 year	Tetralogy of Fallot Repair	2	100
P14	7 months	6.2	30 days -1 year	Ventricular septal defect repair	2	99
P15	11 months	6	30 days -1 year	Damus-Kaye-Stansel anastomosis, Glenn shunt and atrial septectomy	6	88
P16	4 months	4.4	30 days -1 year	Norwood operation	6	79
P17	1 year 11 months	10.6	1-5 years	Ventricular septal defect repair	1	90
P18	2 years 1months	11.2	1-5 years	Ventricular septal defect repair	2	75
P19	1 year 3 months	10.3	1-5 years	Ventricular septal defect repair	2	100
P20	4 years 6 months	16.9	1-5 years	Fontan operation	3	83
P21	1 year 2 months	7.1	1-5 years	Glenn shunt	2	72
P22	16 years 5 months	57.7	10-16 years	Mitral valve repair	3	98
P23	15 years 2 months	55.6	10-16 years	Aortic valve repair	3	100
P24	15 years 4 months	39.4	10-16 years	Aortic valve repair	3	99
P25	10 years 3 months	31.6	10-16 years	Resection of subaortic membrane	2	100
P26	14 years	57.7	10-16 years	Pulmonary valve replacement	2	96
P27	13years 11months	47	10-16 years	Ventricular septal defect repair	2	98
P28	15 years 6 months	31.6	10-16 years	Aortic valve repair/replacement	3	98

*Table 7 Table to summarise pre-operative characteristics of the surgical group: age, weight at operation, recruitment group, type of surgical procedure, RACHS risk category and preoperative oxygen saturation (on room air).*RACHS-1 categorises the risk of hospital mortality according to the type of procedure. The categories range from 1 from (low-risk procedures with mortality of around 0.4%) to 6 (high risk, the morality of 47.7%) (Jenkins et al. 2002)*

Patient	Age	Weight (kg)	Group	Catheter procedure	Preoperative oxygen saturation (%)
P29	15 years 5 months	76.9	10-16 years	Electrophysiology study and ablation	96
P30	15 years 8 months	64.8	10-16 years	Electrophysiology study and ablation	98
P31	11 years 7 months	39.5	10-16 years	Electrophysiology study	98
P32	13 years 5 months	33.3	10-16 years	Electrophysiology study	98
P33	14 years 1 months	102.2	10-16 years	Electrophysiology study	97
P34	13 years 8 months	64	10-16 years	Electrophysiology study	96
P35	16 years 1 months	68.8	10-16 years	Electrophysiology study	99
P36	13 years 3 months	55.9	10-16 years	Ventricular septal defect closure	97

Table 8 Table to summarise patient characteristics for the catheter group: age, weight, recruitment group, type of procedure and preoperative saturations

Patient	Group	Procedure	Procedure time (hours)	CPB time (min)	AoX time (min)	Temperature end of CPB (Celsius)	Aortic cross clamp (degrees Celsius)
P1	<30 days	Transposition of great arteries repair	6:54	206	134	36.6	33.7
P2	<30 days	Transposition of great arteries repair	6:35	209	123	36.4	30
P3	<30 days	Transposition of great arteries repair	6:45	193	113	36.7	36.6
P4	<30 days	Right ventricle to pulmonary artery conduit	4:05	66	no cross clamp	36.3	36.3
P5	<30 days	Interrupted aortic arch repair	6:23	120	59	37	18.9
P6	30 days - 1 year	Ventricular septal defect repair	6:20	171	119	36.6	32.9
P7	30 days - 1 year	Tetralogy of Fallot Repair	4:20	125	75	36.1	34.1
P8	30 days - 1 year	Aortic valve repair	4:50	106	55	36.7	34.8
P9	30 days - 1 year	Left atrioventricular valve repair	3:40	52	35	36.5	36.5
P10	30 days - 1 year	Tetralogy of Fallot Repair	5:13	120	77	36.1	36.1
P11	30 days - 1 year	Glenn shunt and diaphragm plication	1:45	77	no cross clamp	36.3	-
P12	30 days - 1 year	Glenn shunt	5:00	54	9	36.7	-
P13	30 days - 1 year	Tetralogy of Fallot Repair	4:28	60	40	-	36.5
P14	30 days - 1 year	Ventricular septal defect repair	5:10	132	113	36.7	36.6
P15	30 days - 1 year	DKS anastomosis, Glenn shunt and atrial septectomy	7:09	164	78	36.7	28
P16	30 days - 1 year	Norwood operation	9:48	200	85	36.2	18.9
P17	1-5 years	Ventricular septal defect repair	5:00	104	89	36.4	34.9
P18	1-5 years	Ventricular septal defect repair	6:52	163	129	36.5	33
P19	1-5 years	Ventricular septal defect repair	3:40	83	64	36.5	36.5
P20	1-5 years	Fontan operation	5:27	110	no cross clamp	-	-
P21	1-5 years	Glenn shunt	6:03	159	25	36.9	-
P22	10-16 years	Mitral valve repair	5:20	68	46	36	36.4
P23	10-16 years	Aortic valve repair	5:52	124	99	36.7	35.5
P24	10-16 years	Aortic valve repair	6:03	128	83	35	-
P25	10-16 years	Resection of subaortic membrane	3:20	40	27	36.9	34.9
P26	10-16 years	Pulmonary valve replacement	4:18	57	no cross clamp	35.5	-
P27	10-16 years	Ventricular septal defect repair	3:35	63	47	36.6	36.6
P28	10-16 years	Aortic valve repair/replacement	8:40	243	236	36.5	36.5

Table 9 Table to summarise the surgical procedure times, the aortic cross-clamp and CPB times, the temperature of the CPB (end of by-pass and/or aortic cross-clamp removal). Missing data are marked as "-".

Patient	Group	Procedure	Procedure time (hours)
P29	10-16 years	Electrophysiology study and ablation	1:35
P30	10-16 years	Electrophysiology study and ablation	1:46
P31	10-16 years	Electrophysiology study	4:11
P32	10-16 years	Electrophysiology study	2:26
P33	10-16 years	Electrophysiology study	2:48
P34	10-16 years	Electrophysiology study	3:00
P35	10-16 years	Electrophysiology study	2:30
P36	10-16 years	Ventricular septal defect closure	2:30

Table 10 Table to summarise the procedures times for the catheter group

Patient	Group	Procedure	Adrenaline	Noradrenaline	Dopamine	Milrinone
P1	<30 days	Transposition of great arteries repair	✓			✓
P2	<30 days	Transposition of great arteries repair	✓			✓
P3	<30 days	Transposition of great arteries repair	✓			✓
P4	<30 days	Right ventricle to pulmonary artery conduit				✓
P5	<30 days	Interrupted aortic arch repair	✓		✓	
P6	30 days -1 year	Ventricular septal defect repair				✓
P7	30 days -1 year	Tetralogy of Fallot Repair		✓		✓
P8	30 days -1 year	Aortic valve repair				
P9	30 days -1 year	Left atrioventricular valve repair				✓
P10	30 days -1 year	Tetralogy of Fallot Repair			✓	✓
P11	30 days -1 year	Glenn shunt and diaphragm plication			✓	✓
P12	30 days -1 year	Glenn shunt			✓	✓
P13	30 days -1 year	Tetralogy of Fallot Repair			✓	✓
P14	30 days -1 year	Ventricular septal defect repair			✓	✓
P15	30 days -1 year	Damus-Kaye-Stansel, Glenn shunt and atrial septectomy			✓	✓
P16	30 days -1 year	Norwood operation	✓		✓	✓
P17	1-5 years	Ventricular septal defect repair			✓	✓
P18	1-5 years	Ventricular septal defect repair			✓	✓
P19	1-5 years	Ventricular septal defect repair				✓
P20	1-5 years	Fontan operation			✓	✓
P21	1-5 years	Glenn shunt			✓	✓
P22	10-16 years	Mitral valve repair			✓	
P23	10-16 years	Aortic valve repair			✓	✓
P24	10-16 years	Aortic valve repair			✓	✓
P25	10-16 years	Resection of subaortic membrane				
P26	10-16 years	Pulmonary valve replacement				
P27	10-16 years	Ventricular septal defect repair				
P28	10-16 years	Aortic valve repair/replacement		✓		✓

Table 11 Use of vasopressors and inotropes by age group for surgery patients

Patient	Group	Ventilation time (hours)	Preop Hb	Lowest post-op Hb	Preoperative Creatinine	Highest postoperative creatinine	% increase of Creatinine	PICU Stay (days)	Duration admission (days)	Status on discharge
P1	<30 days	5 days 5 hrs 15 mins	133	111	38	64	68%	7	10	Alive
P2	<30 days	4 days 3 hrs 48 mins	175	122	43	51	19%	5	12	Alive
P3	<30 days	8 days 4 hrs 20 mins	147	130	45	73	62%	-	-	Alive
P4	<30 days	6 days 5 hrs 0 mins	82	118	27	53	96%	9	13	Alive
P5	<30 days	12 days 3 hrs 5 mins	154	137	38	67	76%	14	35	Alive
P6	30 days -1 year	1 day 4 hrs 39 mins	101	106	16	23	44%	2	2	Alive
P7	30 days -1 year	2 days 2 hrs 31 mins	134	110	23	30	30%	3	5	Alive
P8	30 days -1 year	1 day 4 hrs 40 mins	117	129	23	28	22%	3		Alive
P9	30 days -1 year	5 hrs 10 mins	111	140	22	23	5%	1	6	Alive
P10	30 days -1 year	7 days 2 hrs 31 mins	124	105	24	101	321%	8	14	Alive
P11	30 days -1 year	24 days 5 hrs 40 mins	144	117	23	20	13%	36	113	Alive
P12	30 days -1 year	11 hrs 34 mins	130	123	22	21	5%	3	21	Alive
P13	30 days -1 year	21 hrs 53 mins	113	80	31	41	32%	2	15	Alive
P14	30 days -1 year	3 days 3 hrs 25 mins	112	106	29	39	34%	4	10	Alive
P15	30 days -1 year	10 hrs 0 mins	151	72	19	22	16%	0	6	Alive

Patient	Group	Ventilation time (hours)	Preop Hb	Lowest post-op Hb	Preoperative Creatinine	Highest postoperative creatinine	% increase of Creatinine	PICU Stay (days)	Duration admission (days)	Status on discharge
P16	30 days -1 year	11 days 22 hrs 9 mins	162	120	25	25	0%	38	43	Alive
P17	1-5 years	2 days 12 hrs 7 mins	158	131	27	62	130%	2	7	Alive
P18	1-5 years	1 day 8 hrs 8 mins	144	92	32	68	113%	37	98	Alive
P19	1-5 years	0 days 10 hrs 55 mins	130	113	23	28	22%	2	6	Alive
P20	1-5 years	6 day 13 hrs 6 mins	189	117	44	60	36%	8	34	Alive
P21	1-5 years	1 day 20 hrs 0 mins	169	106	24	24	0%	2	6	Alive
P22	10-16 years	6 hrs 5 mins	154	108	87	94	8%	1	6	Alive
P23	10-16 years	4 hrs 32 mins	152	94	60	78	30%	3	12	Alive
P24	10-16 years	4 hrs 5 mins	133	101	58	57	2%	2	5	Alive
P25	10-16 years	4 hrs 20 mins	140	94	39	48	23%	1	5	Alive
P26	10-16 years	4 hrs 32 mins	133	109	60	65	8%	1	7	Alive
P27	10-16 years	4 hrs 5 mins	136	100	58	70	21%	1	4	Alive
P28	10-16 years	2 days 4 hrs 20 mins	130	79	43	52	21%	5	20	Alive

Table 12 Table to summarise the relevant clinical outcomes of the different age groups duration of mechanical ventilation, perioperative Hb measurements, perioperative creatinine, PICU stay, the total duration of admission and hospital mortality. Missing data are marked as "- ".

11.6.3 Individual cortisol profiles

In this chapter, I have included cortisol profiles of 34 patients. These profiles are grouped by age group. In the end, I have also included two more profiles that were withdrawn from the study due to the administration of glucocorticoids during the sampling period but, are very interesting to discuss from a mechanistic point of view.

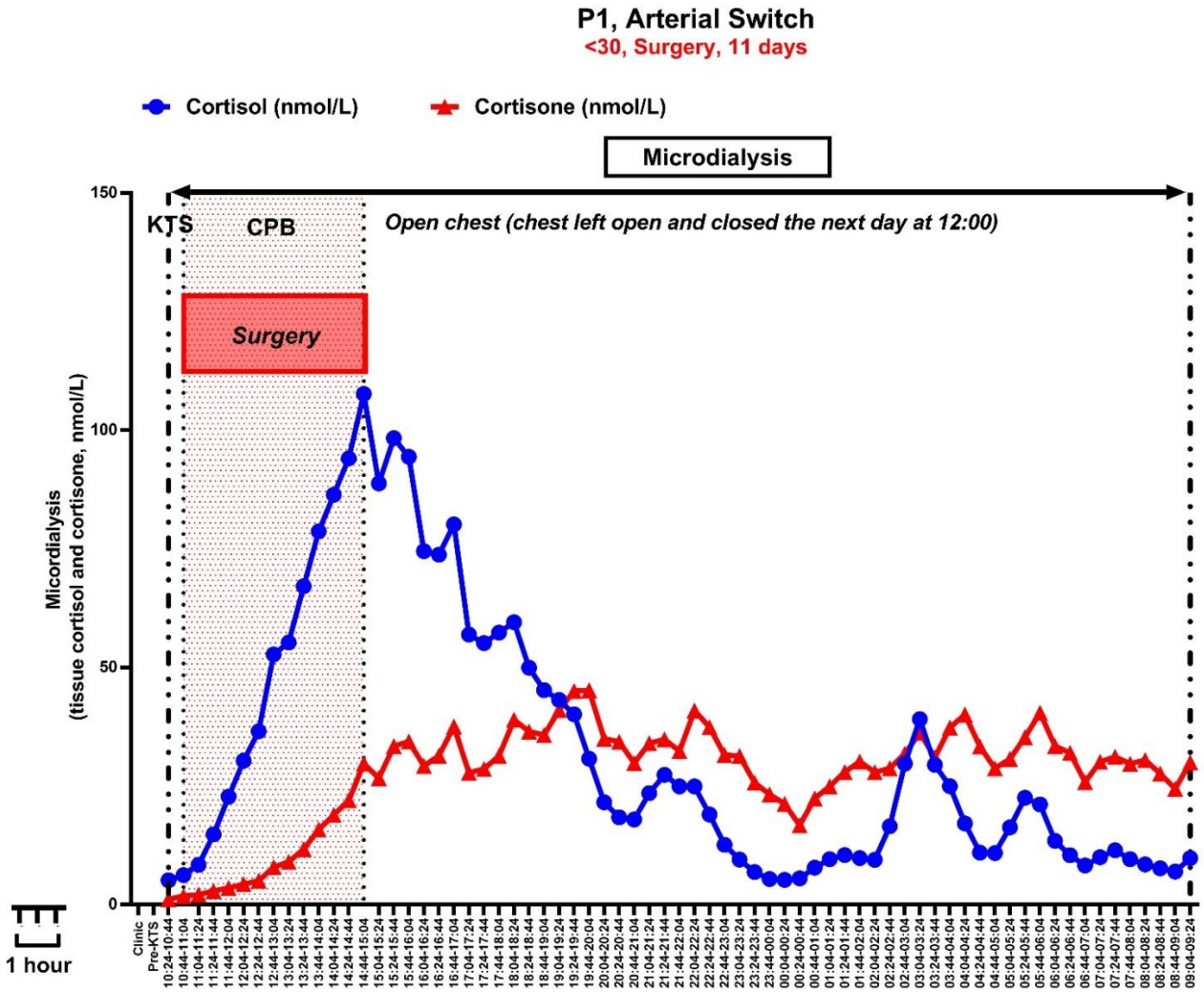

Figure 44 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

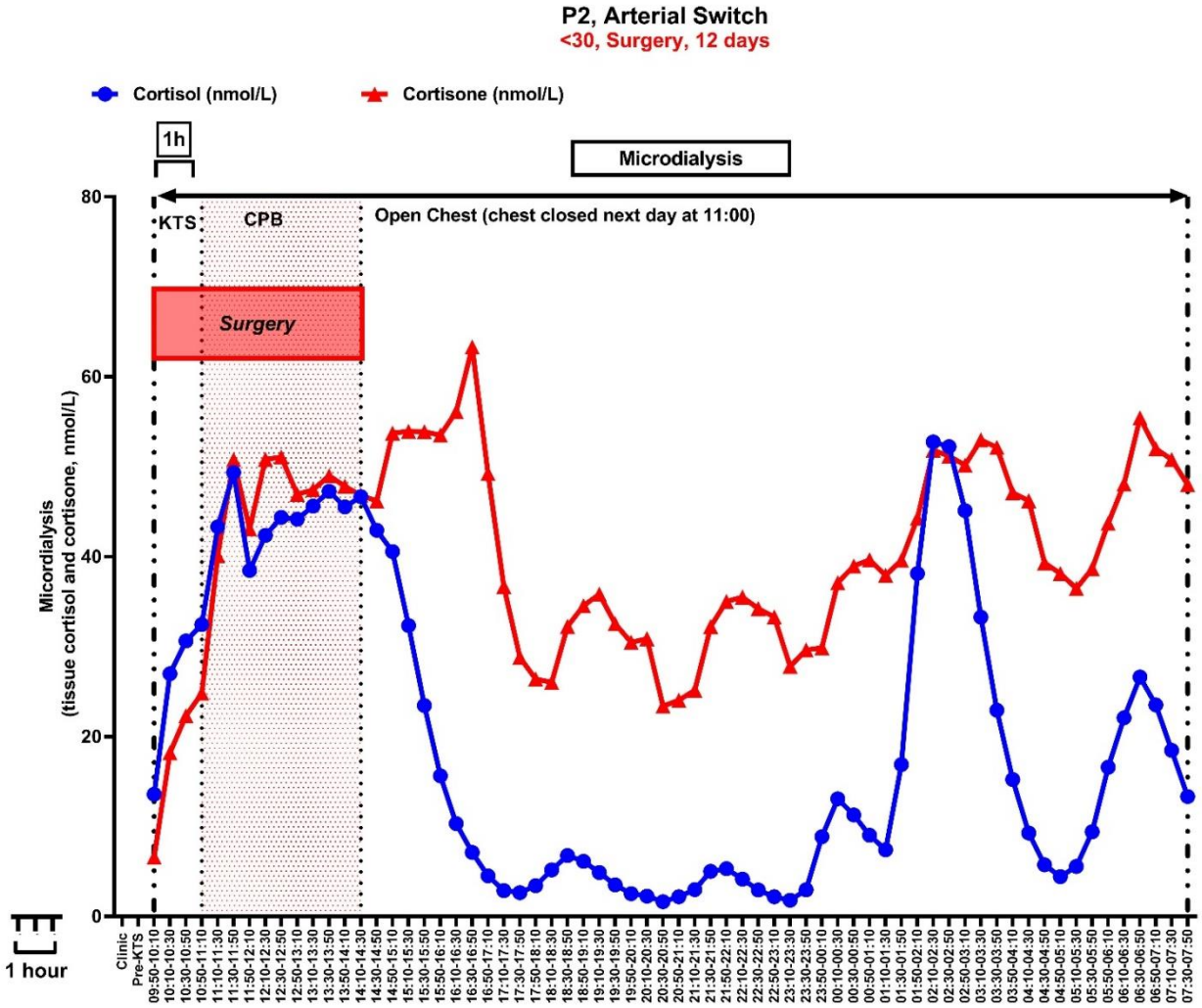

Figure 45 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

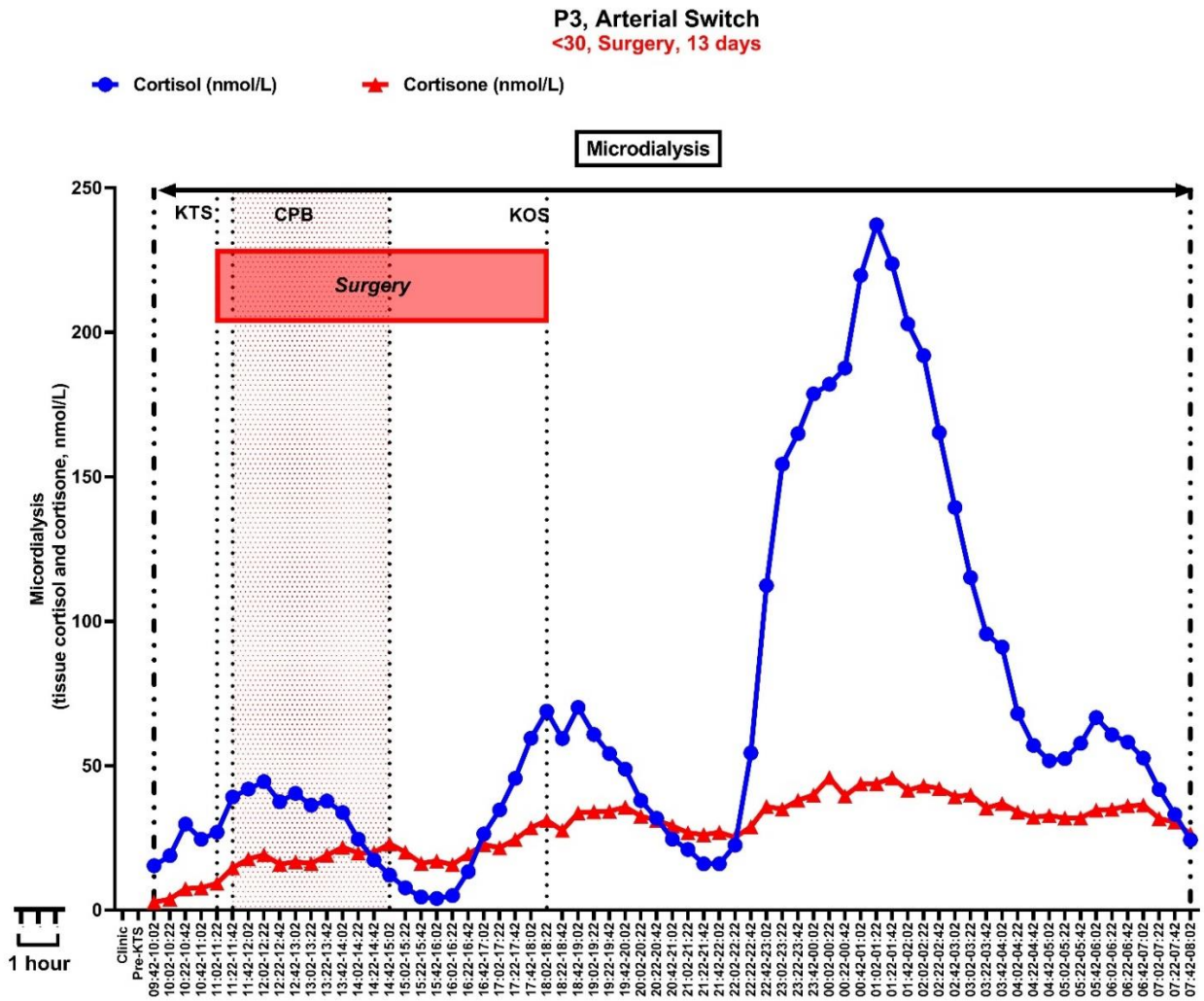

Figure 46 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

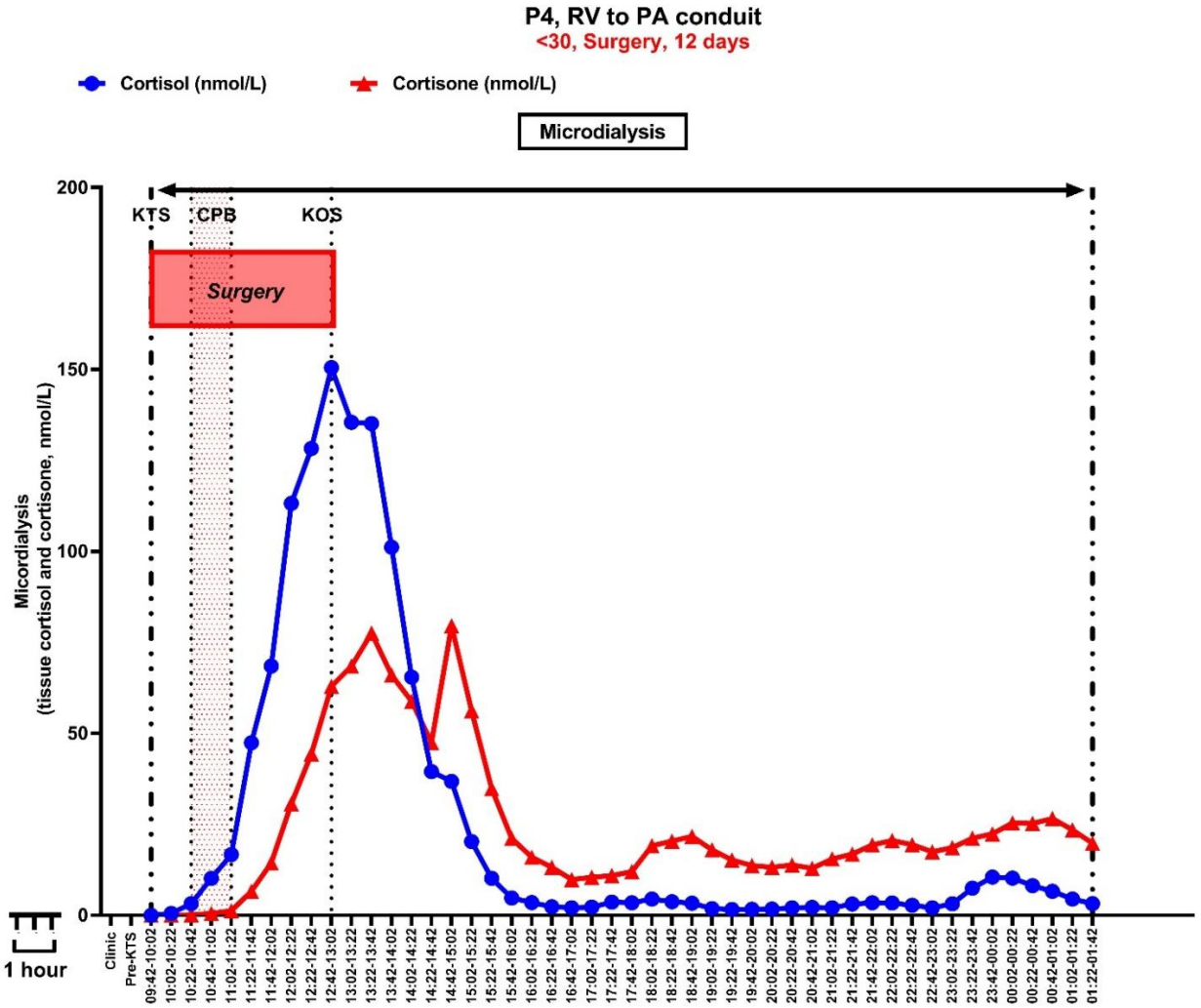

Figure 47 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife off skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

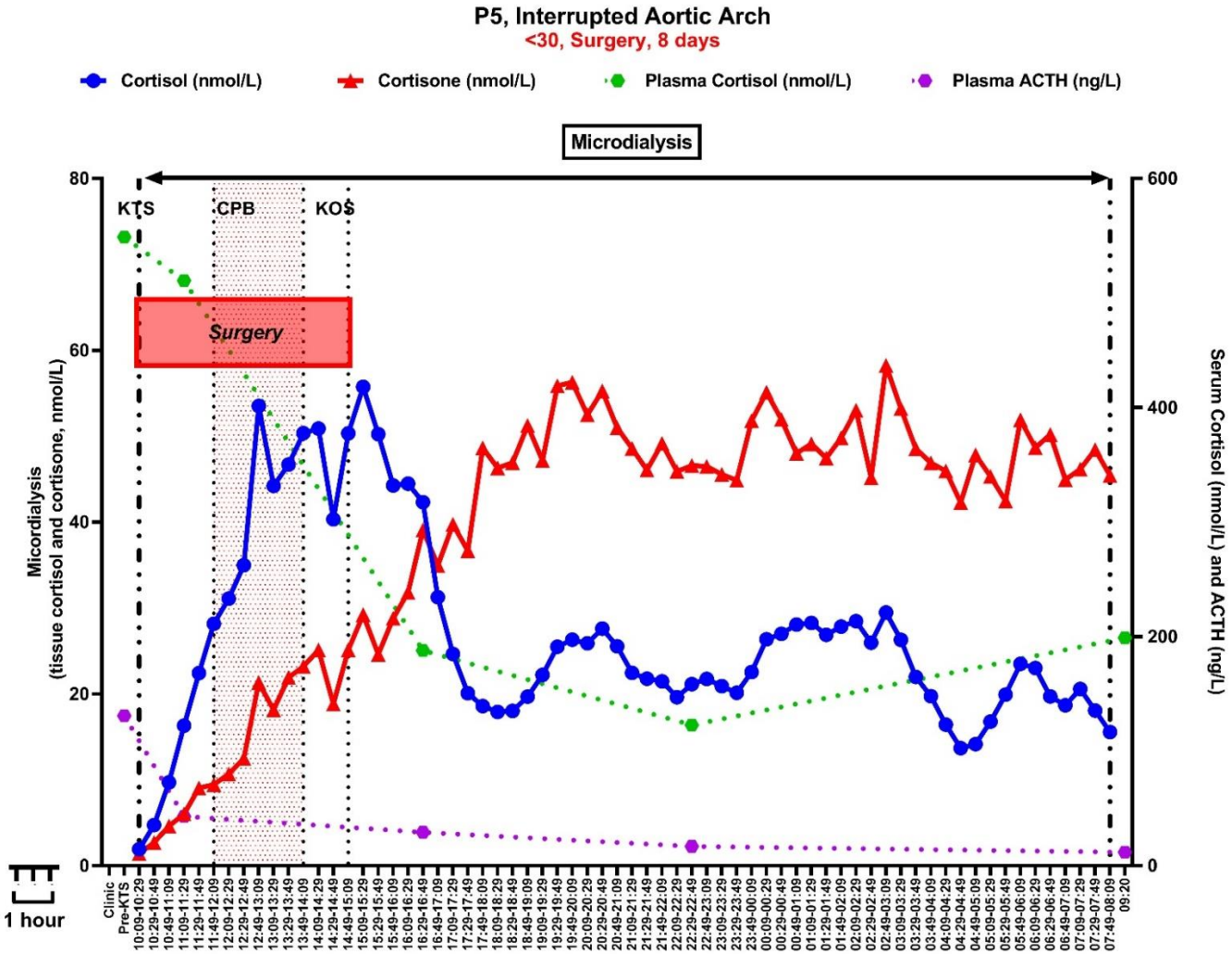

Figure 48 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

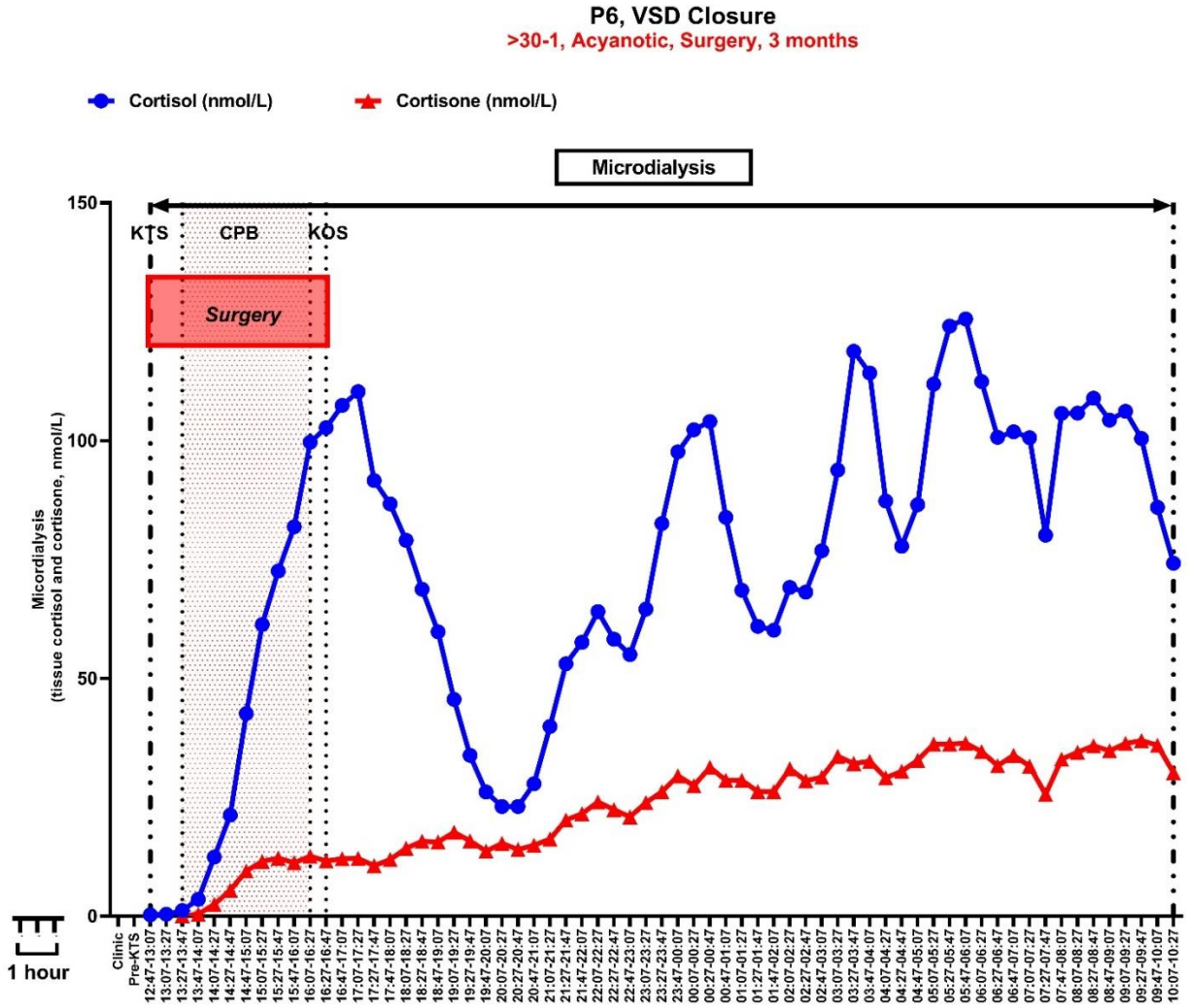

Figure 49 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

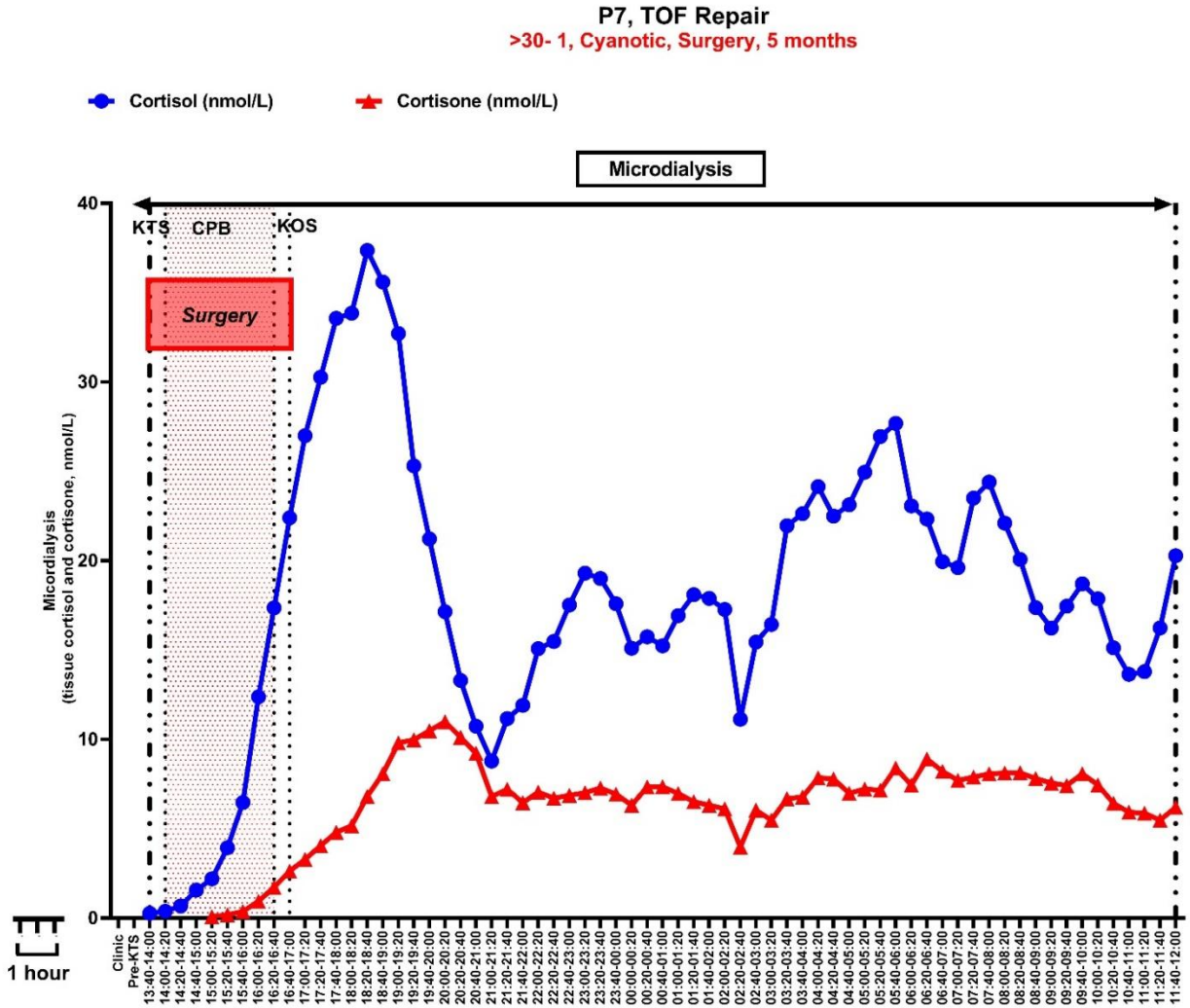

Figure 50 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

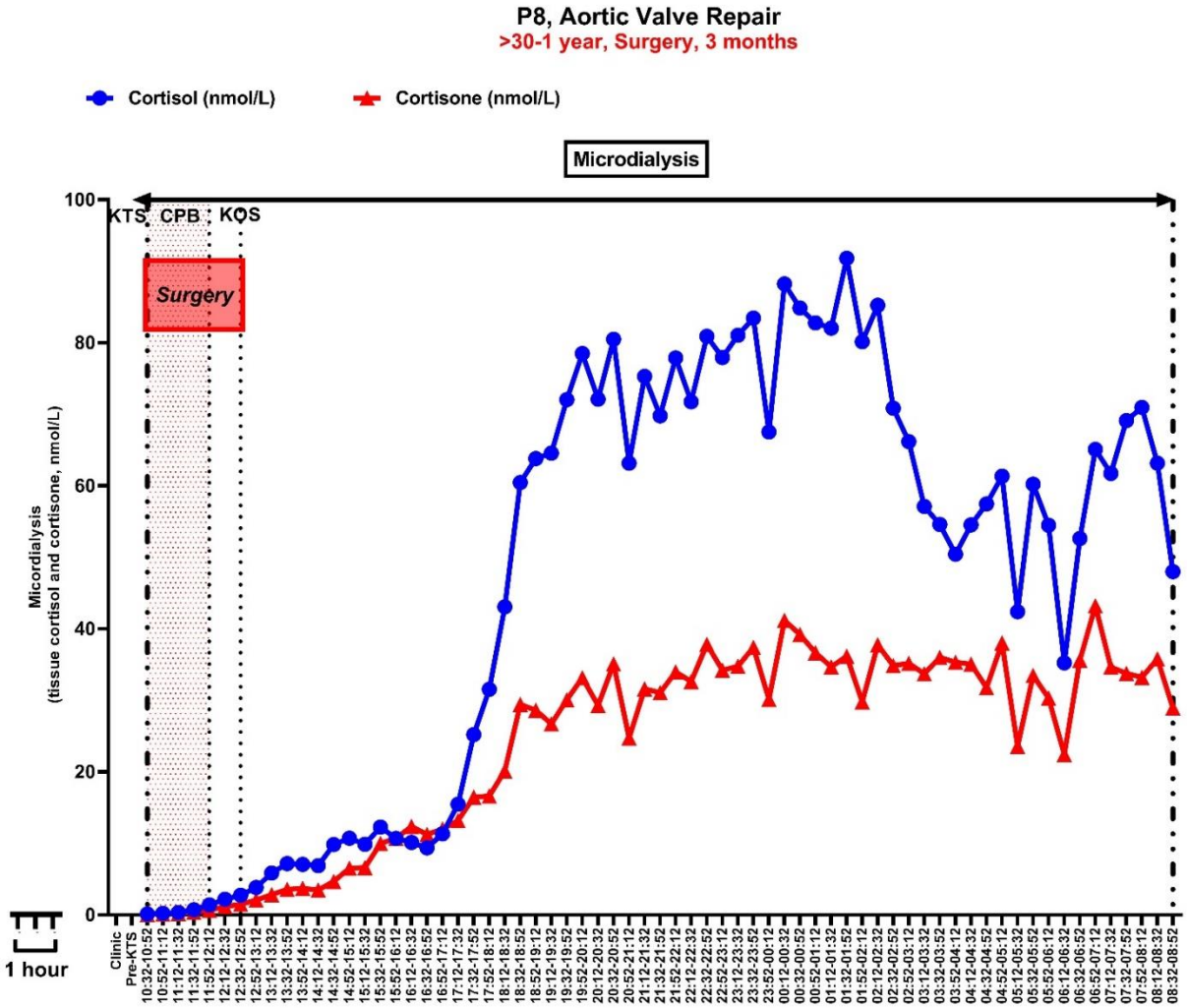

Figure 51 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

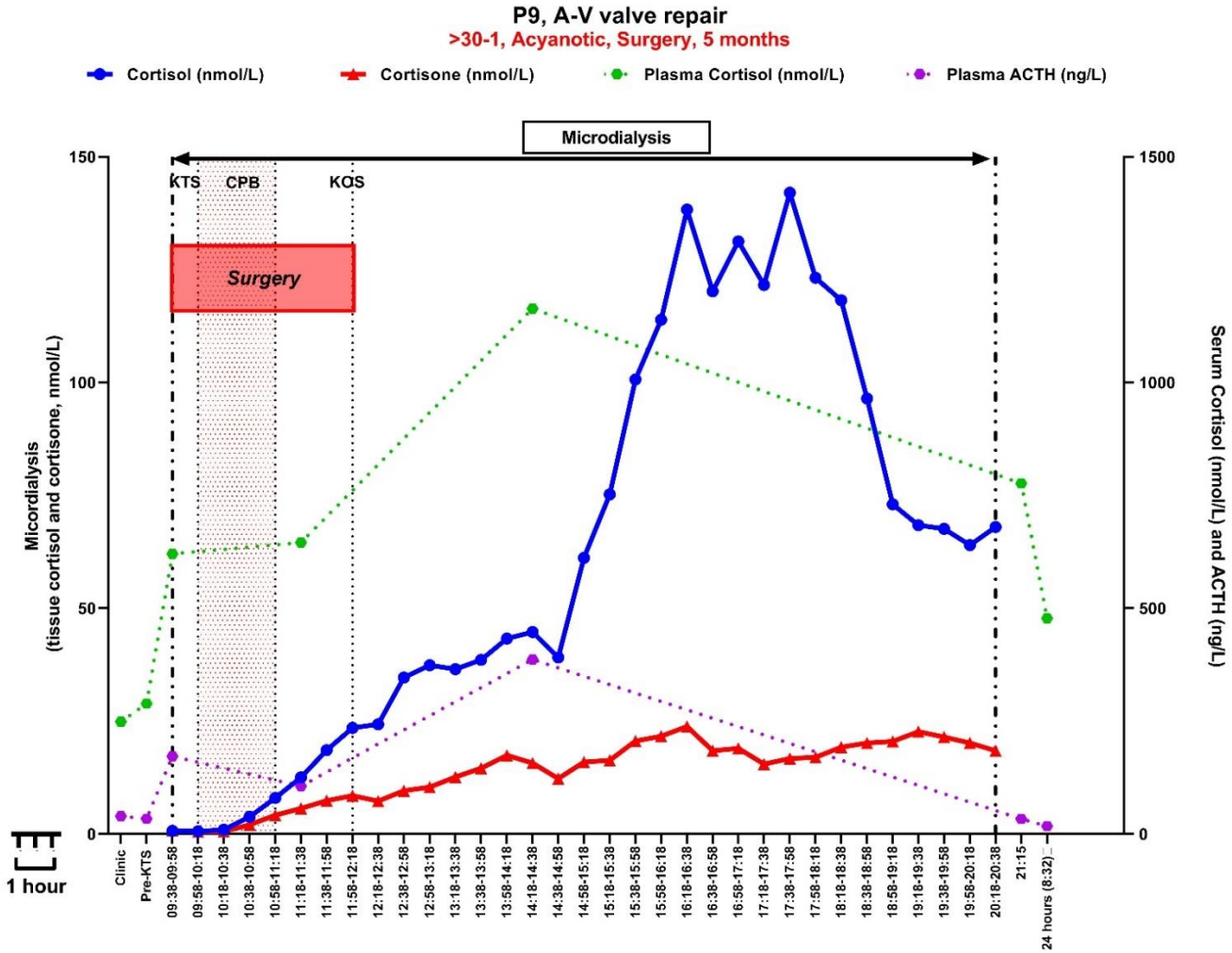

Figure 52 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

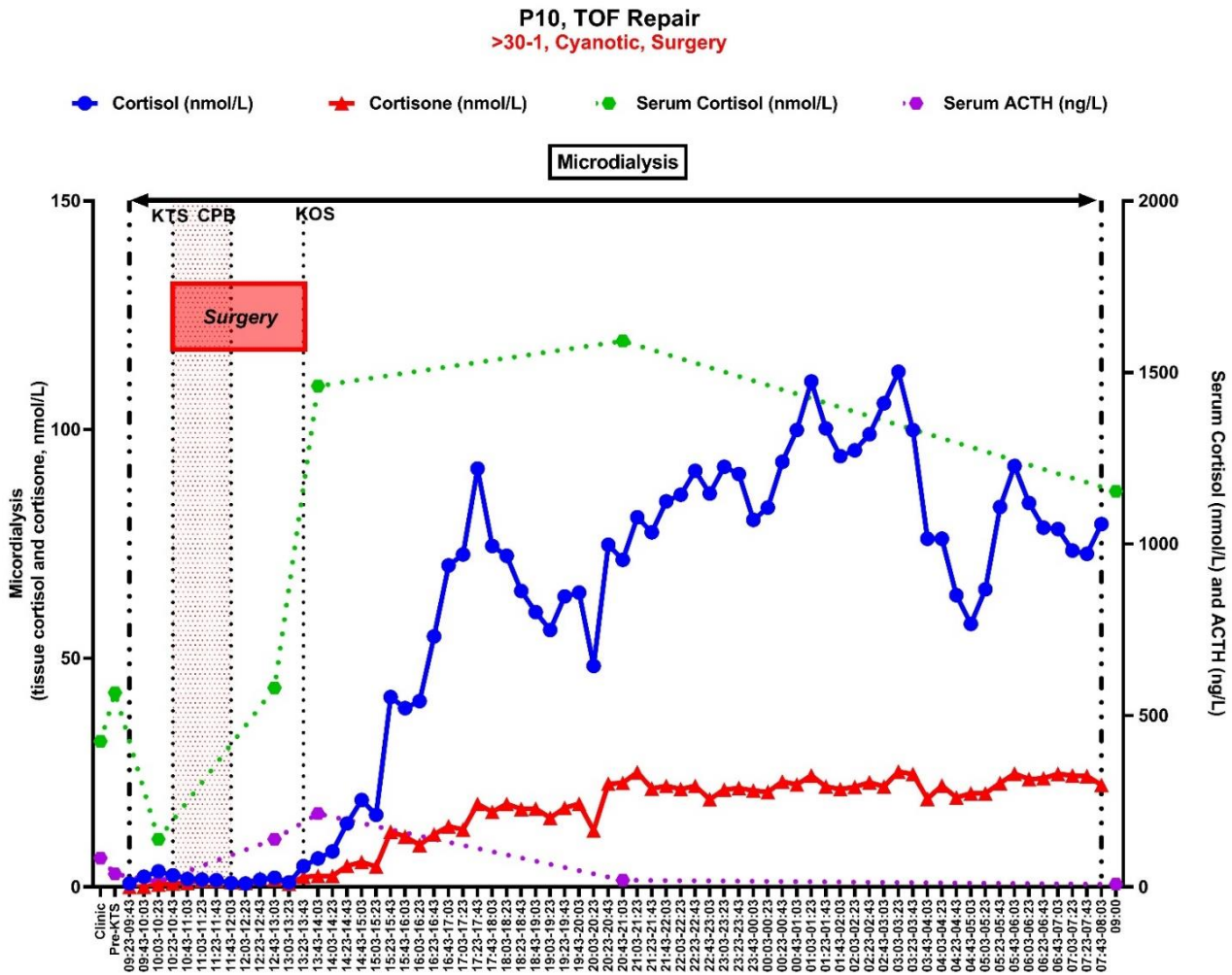

Figure 53 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

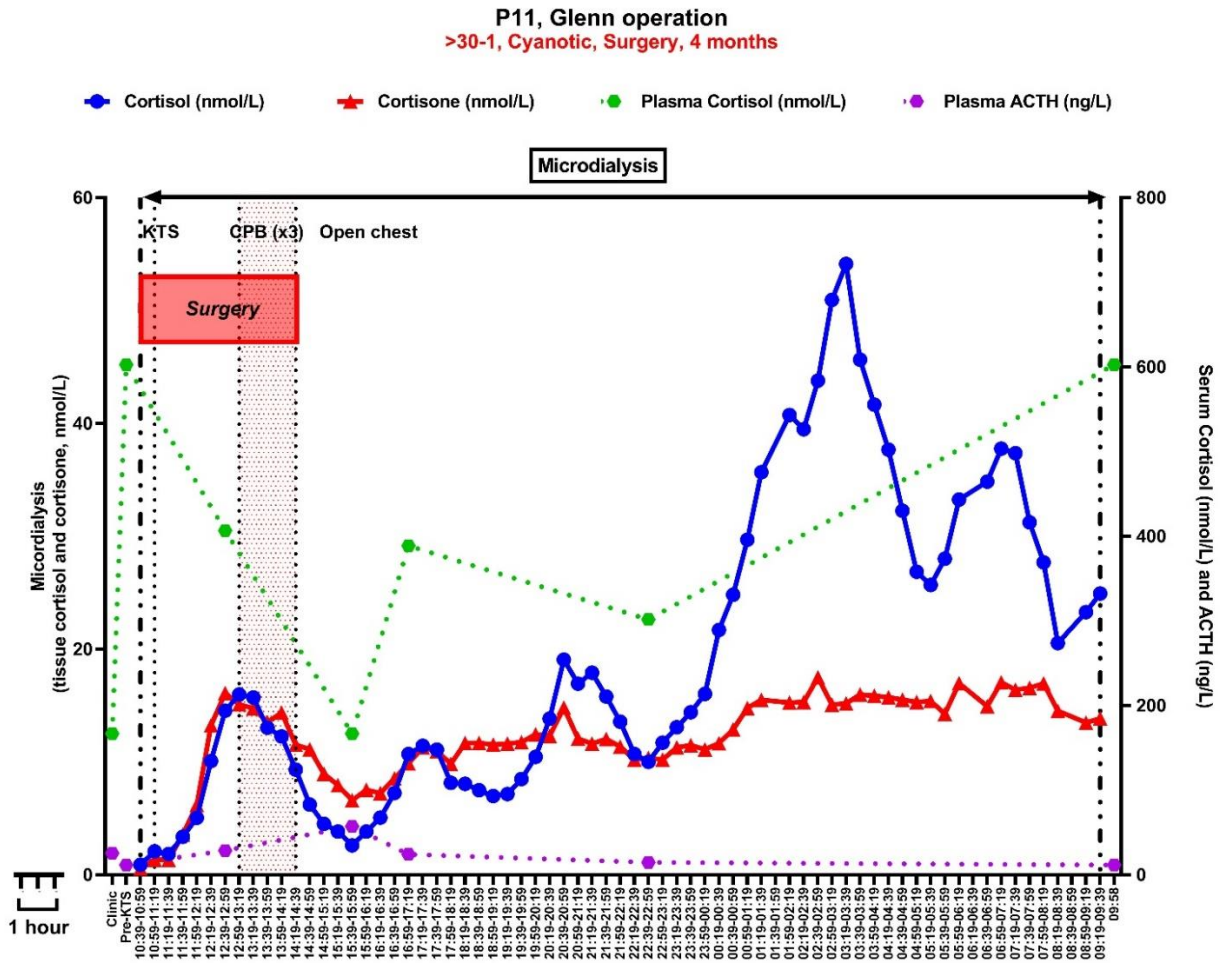

Figure 54 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

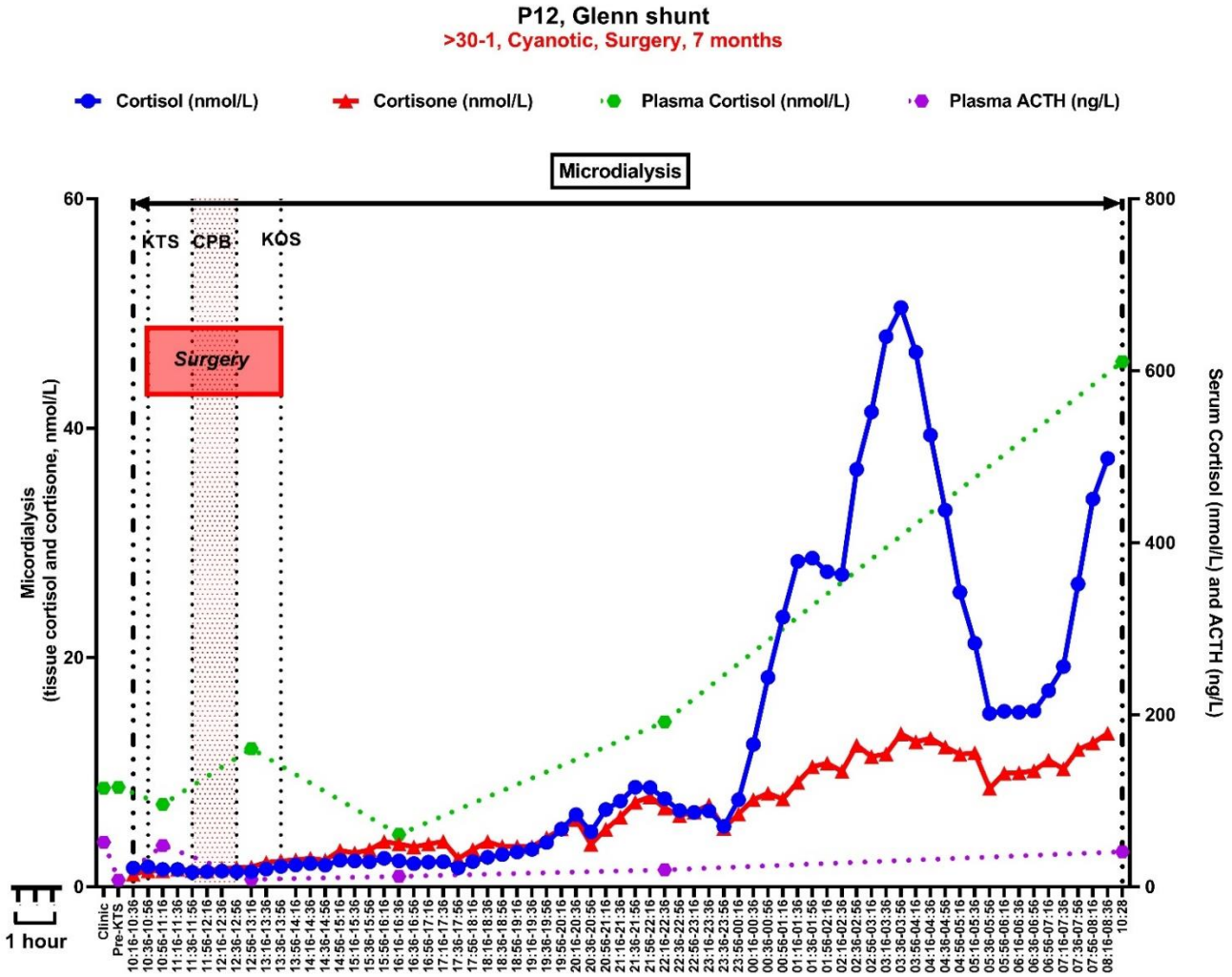

Figure 55 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

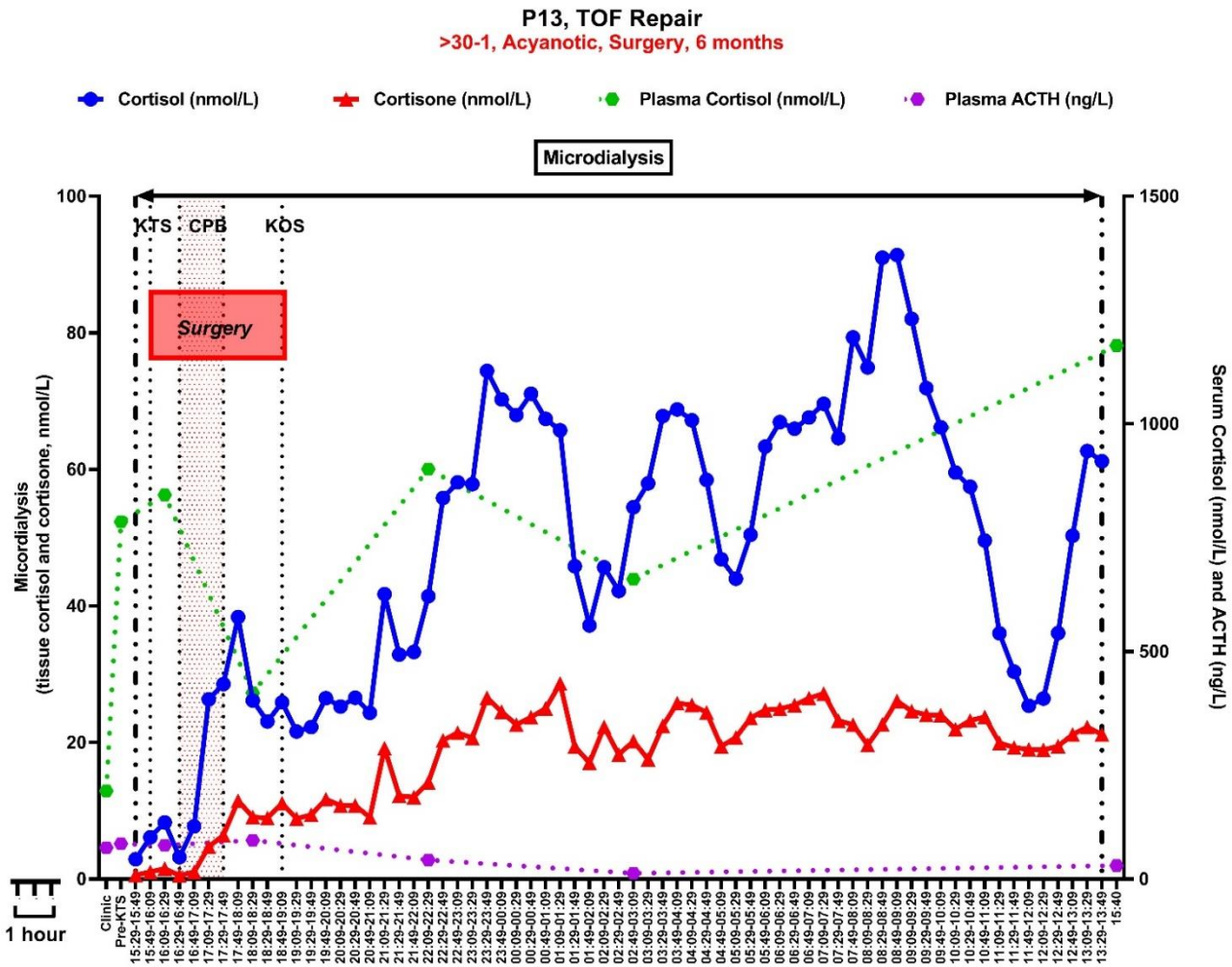

Figure 56 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

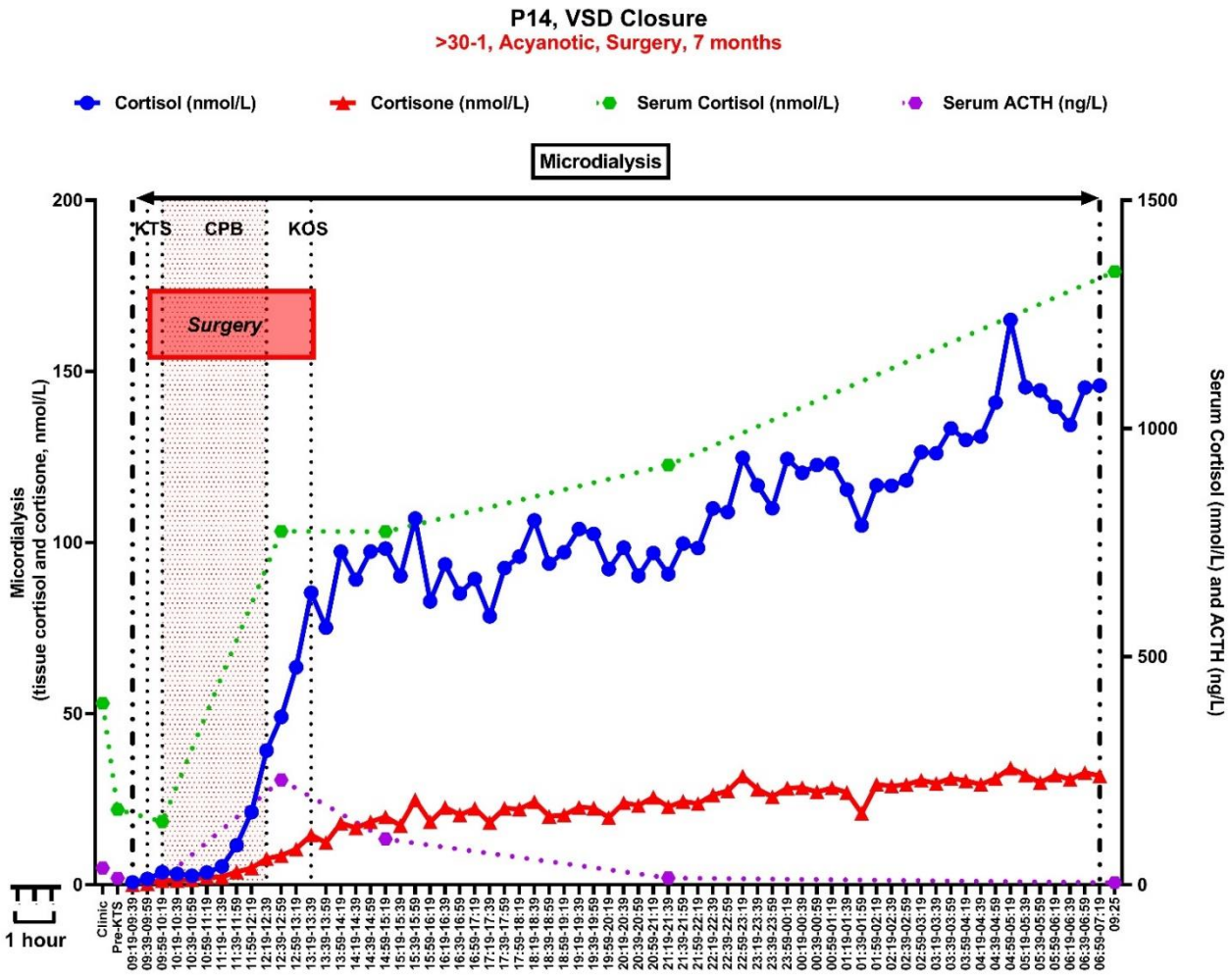

Figure 57 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

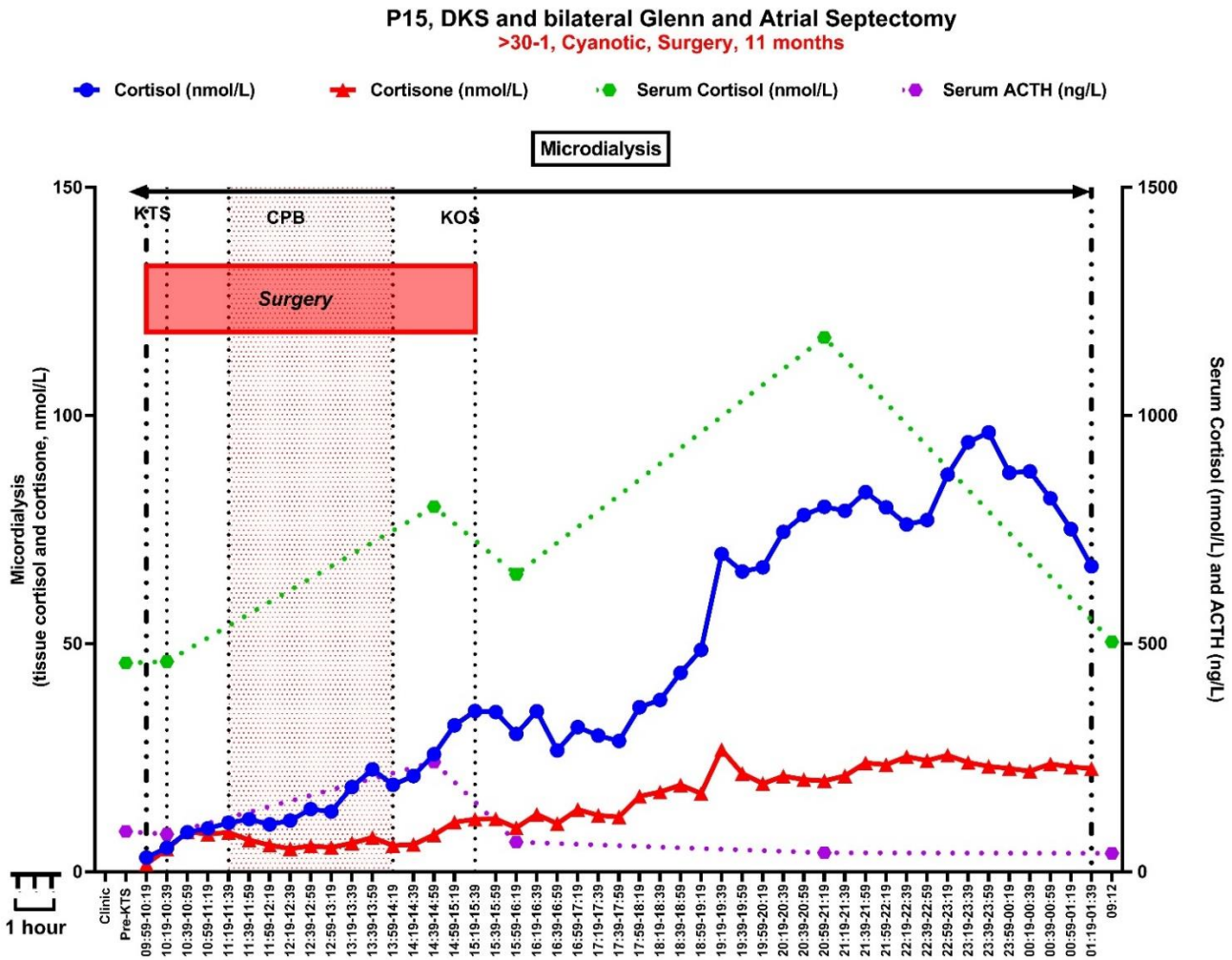

Figure 58 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

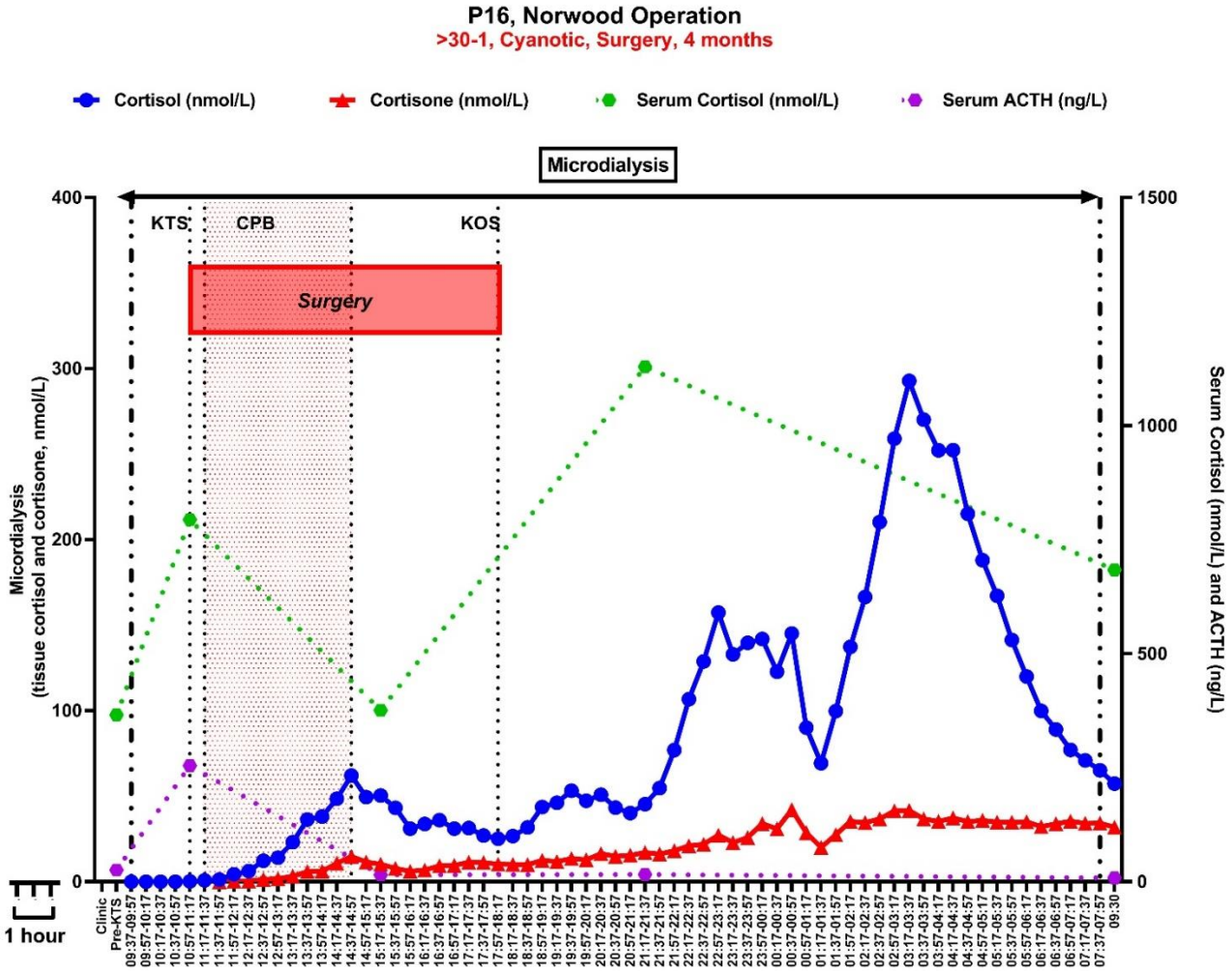

Figure 59 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

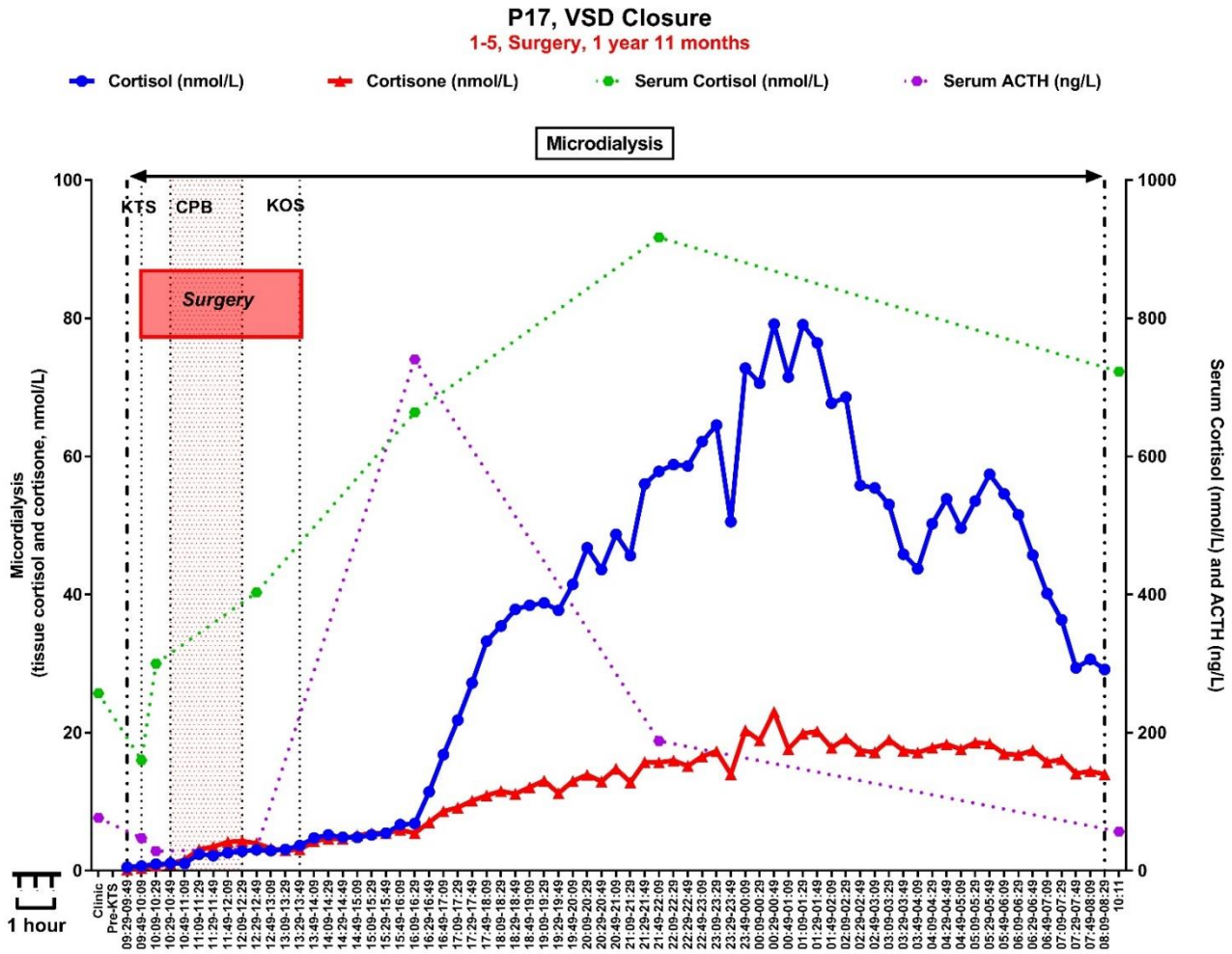

Figure 60 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

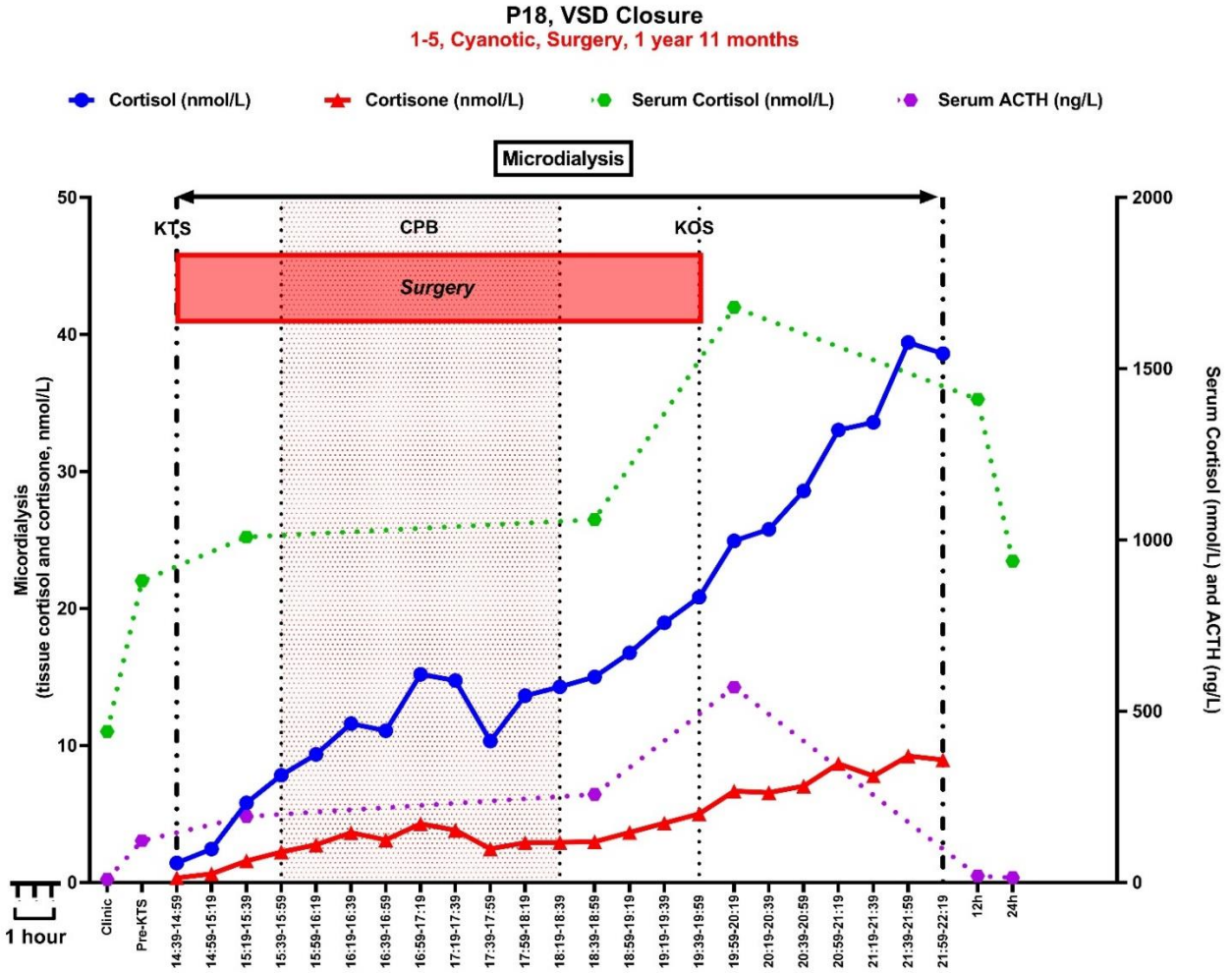

Figure 61 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

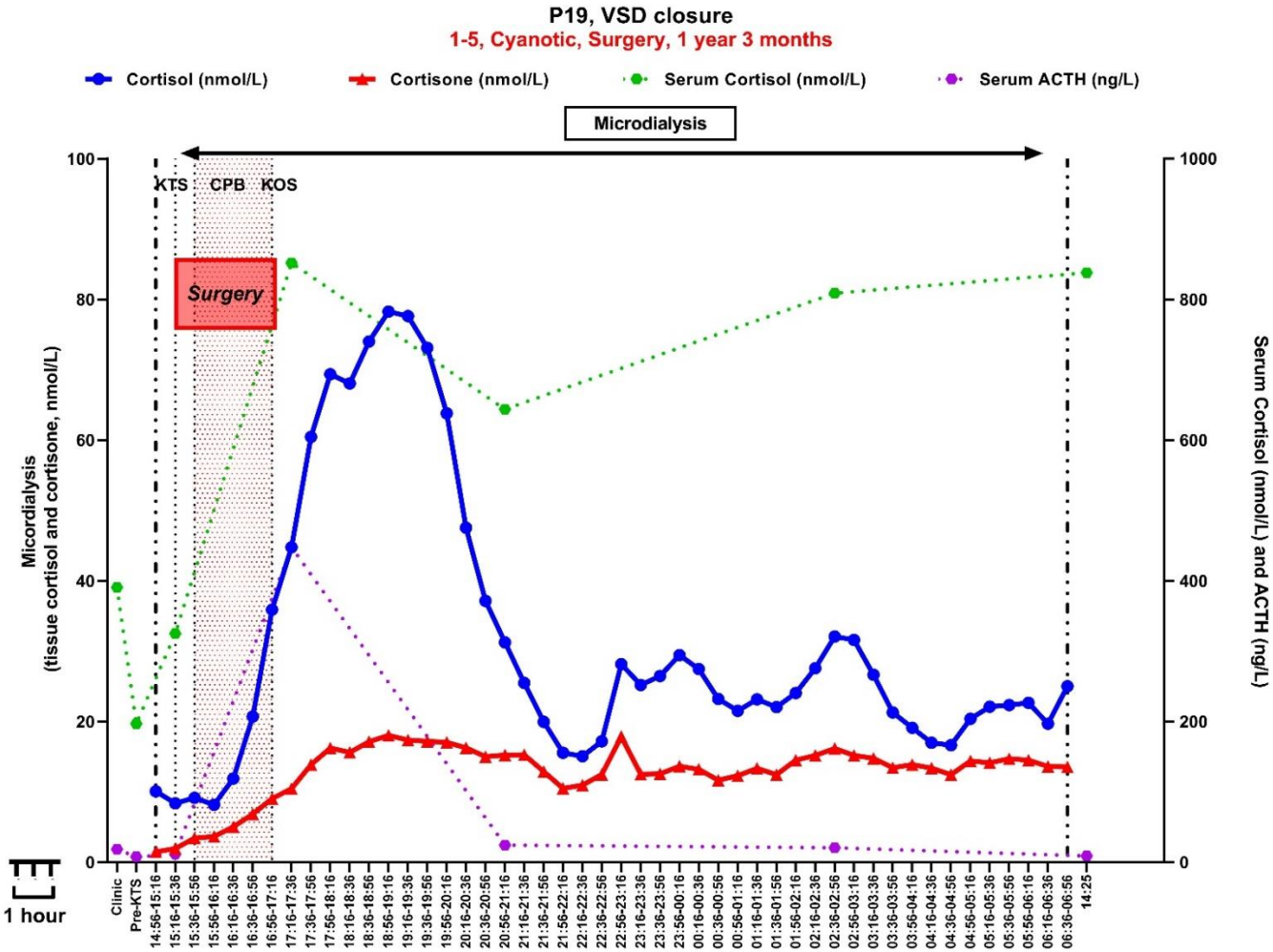

Figure 62 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

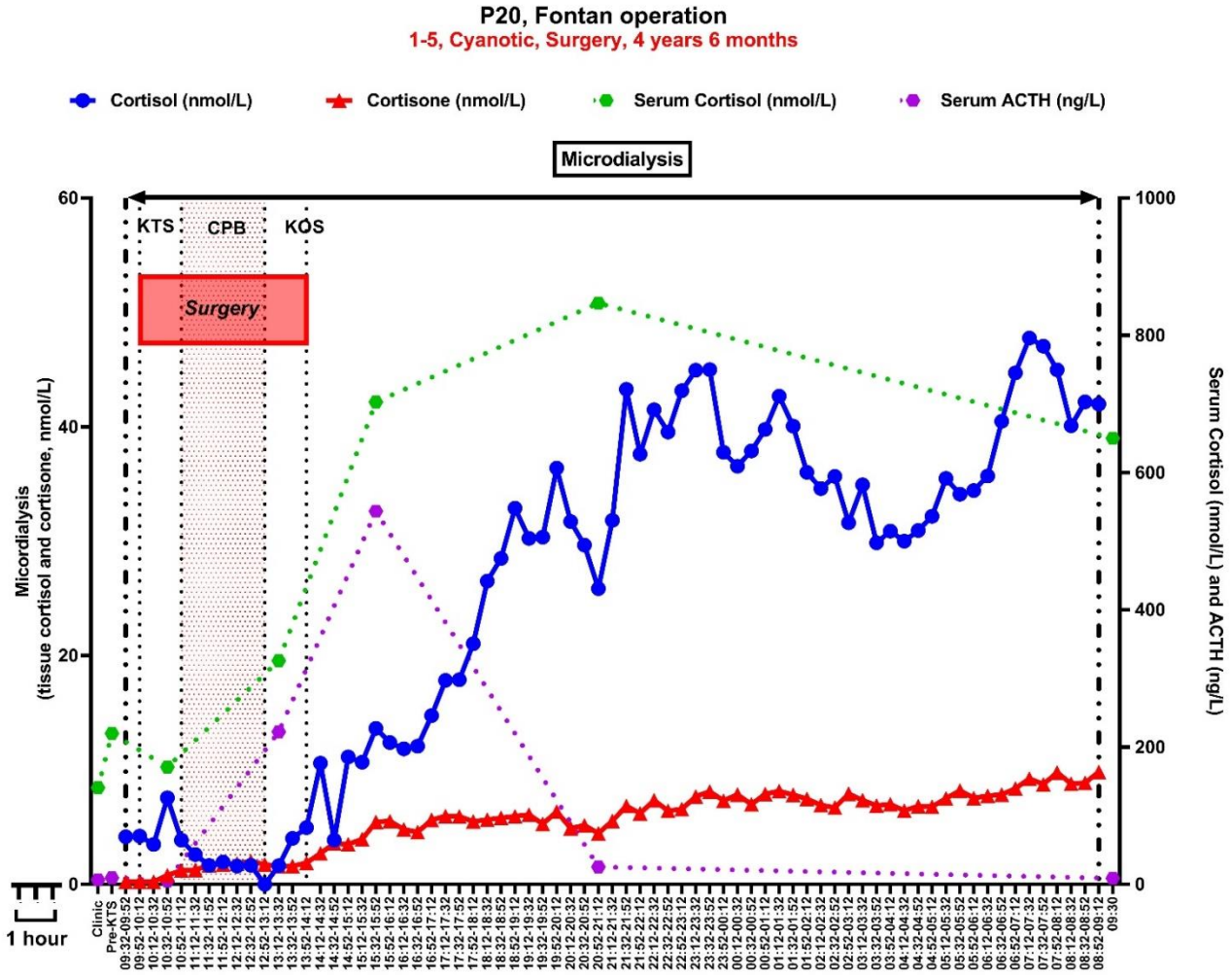

Figure 63 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

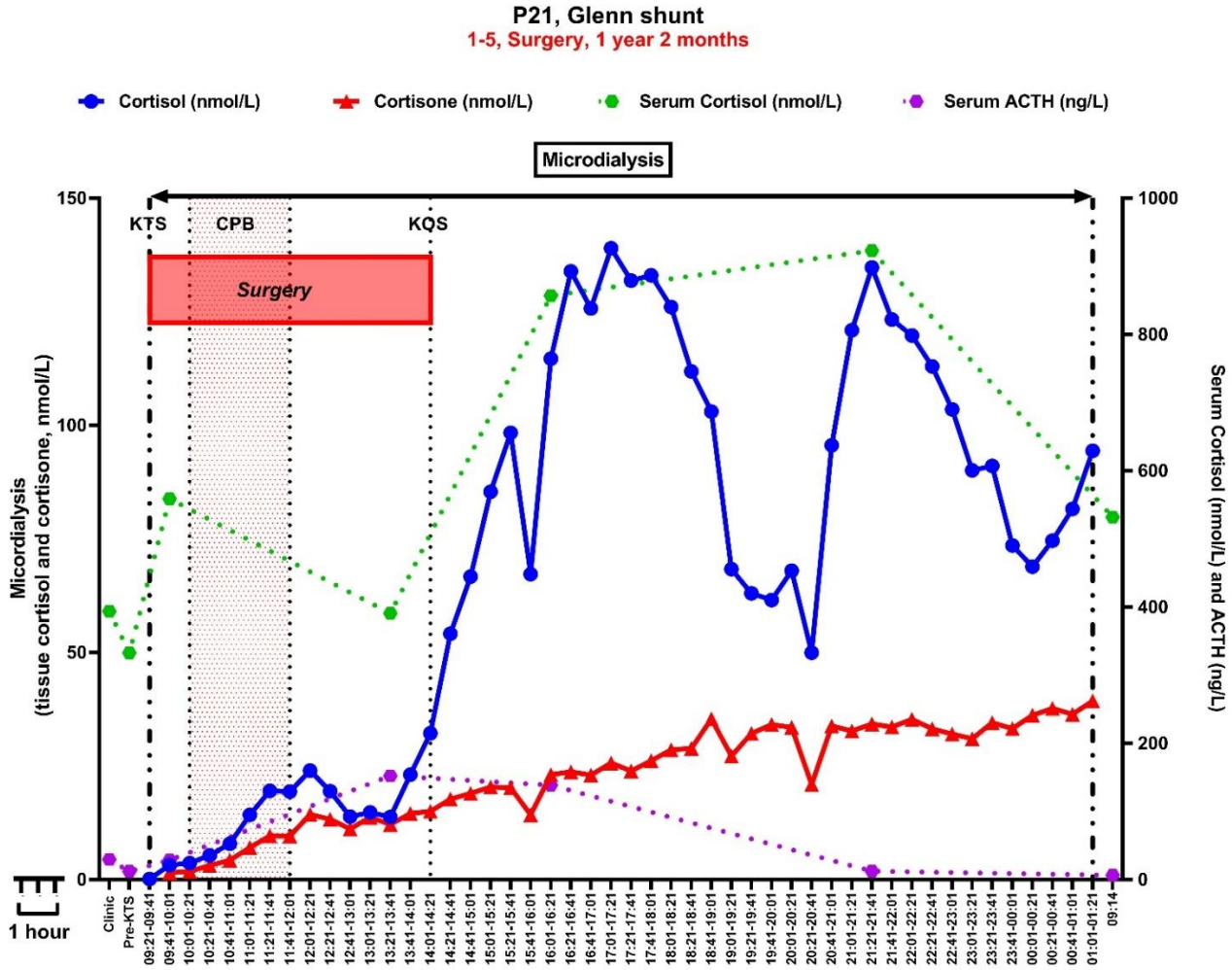

Figure 64 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

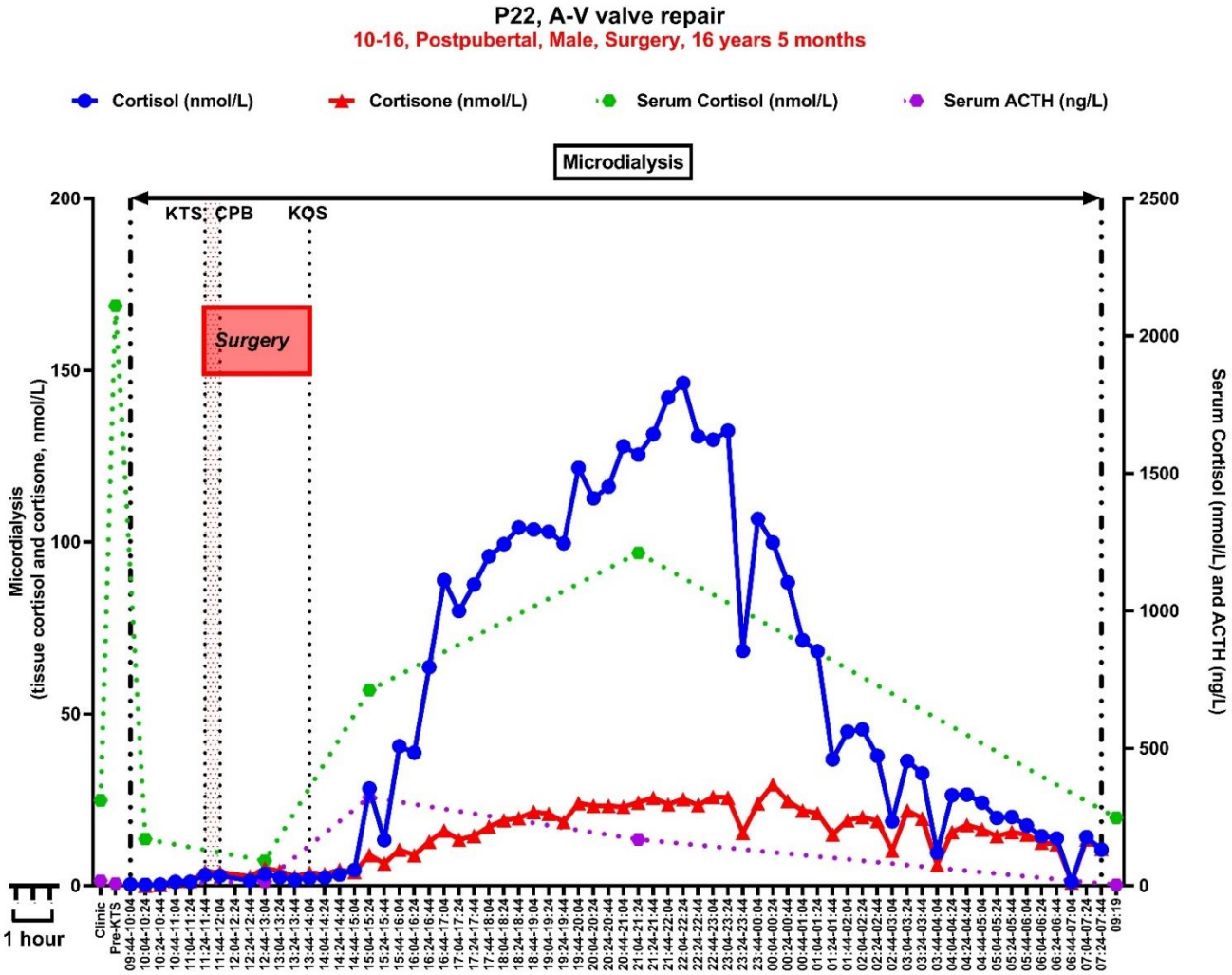

Figure 65 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

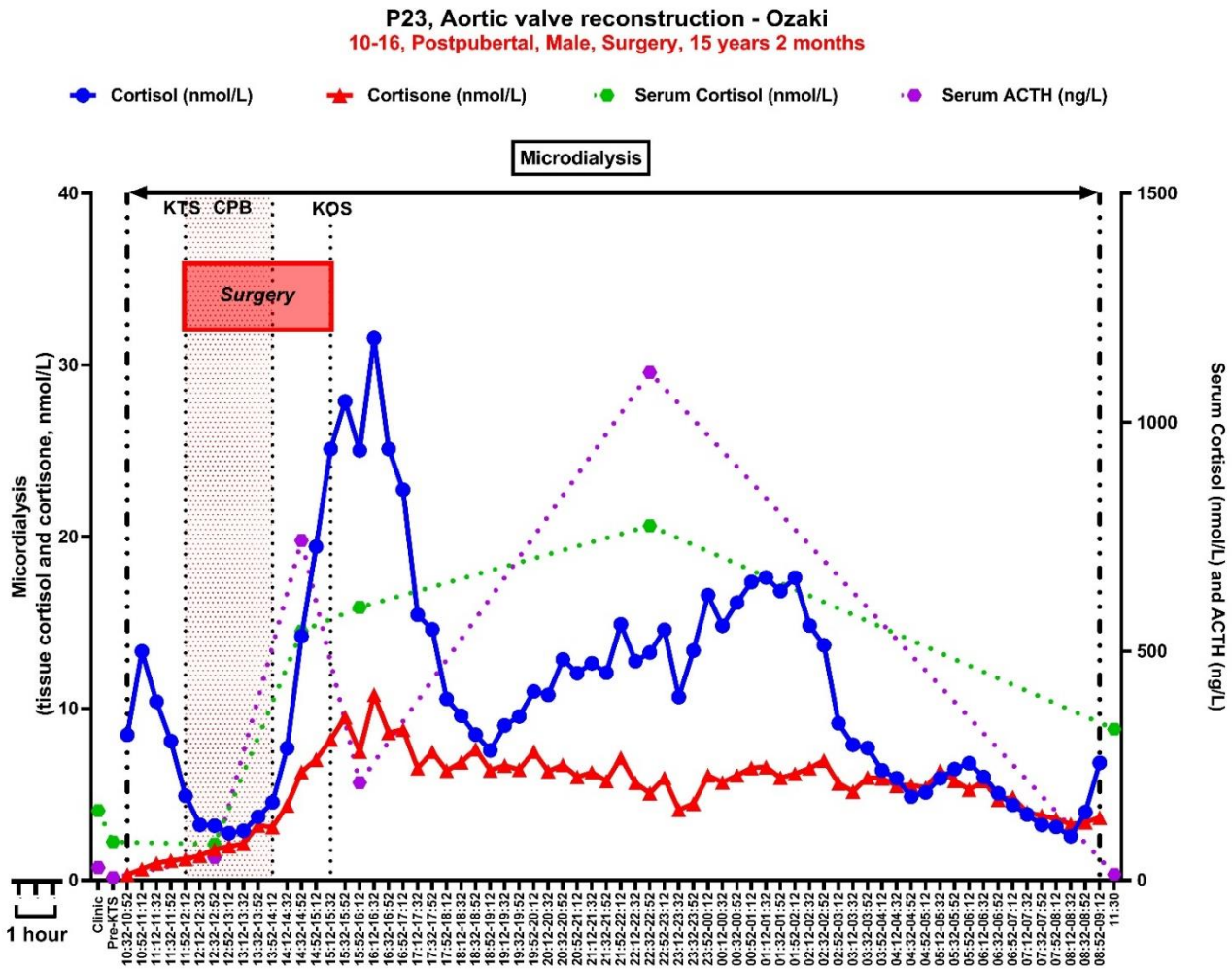

Figure 66 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

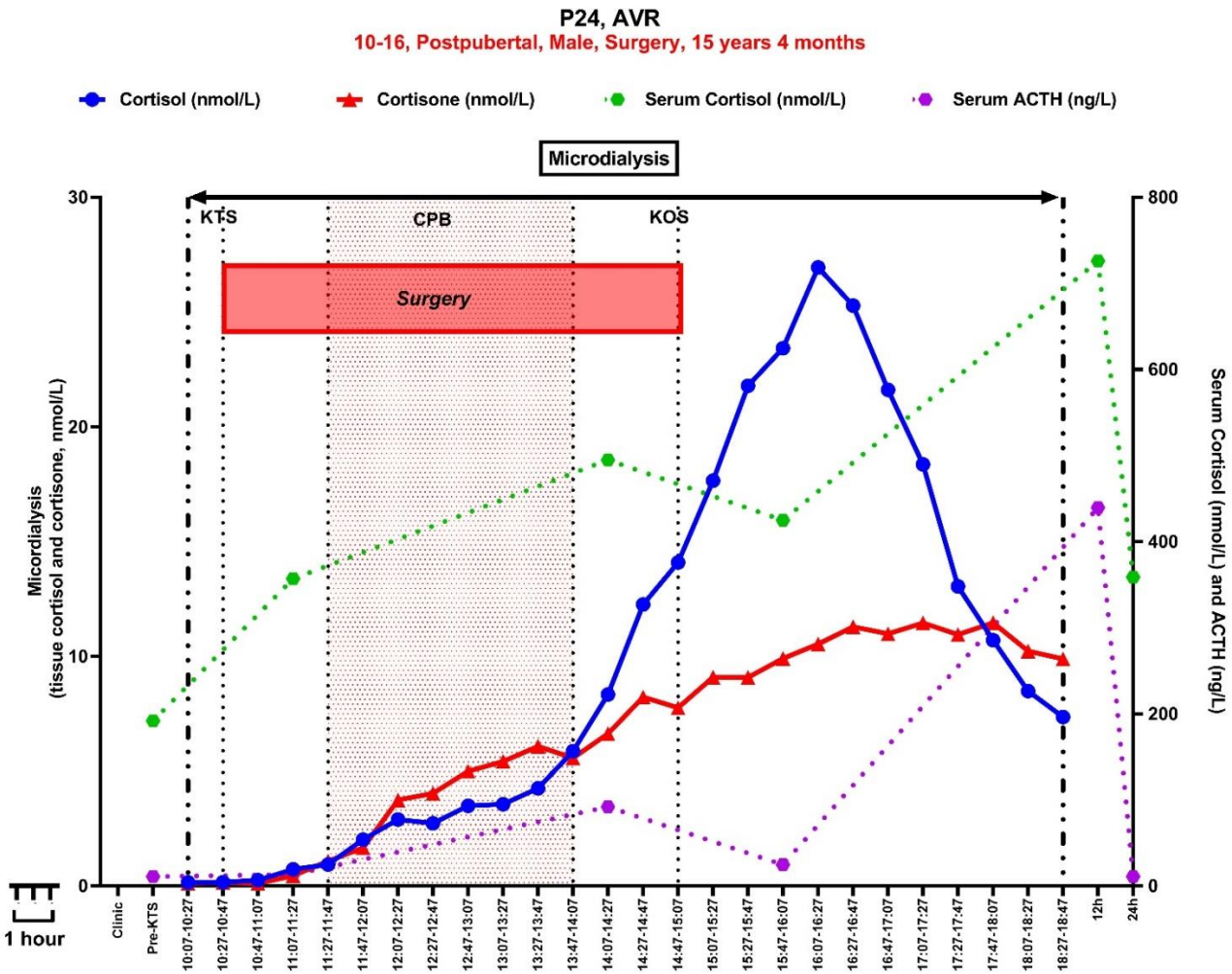

Figure 67 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

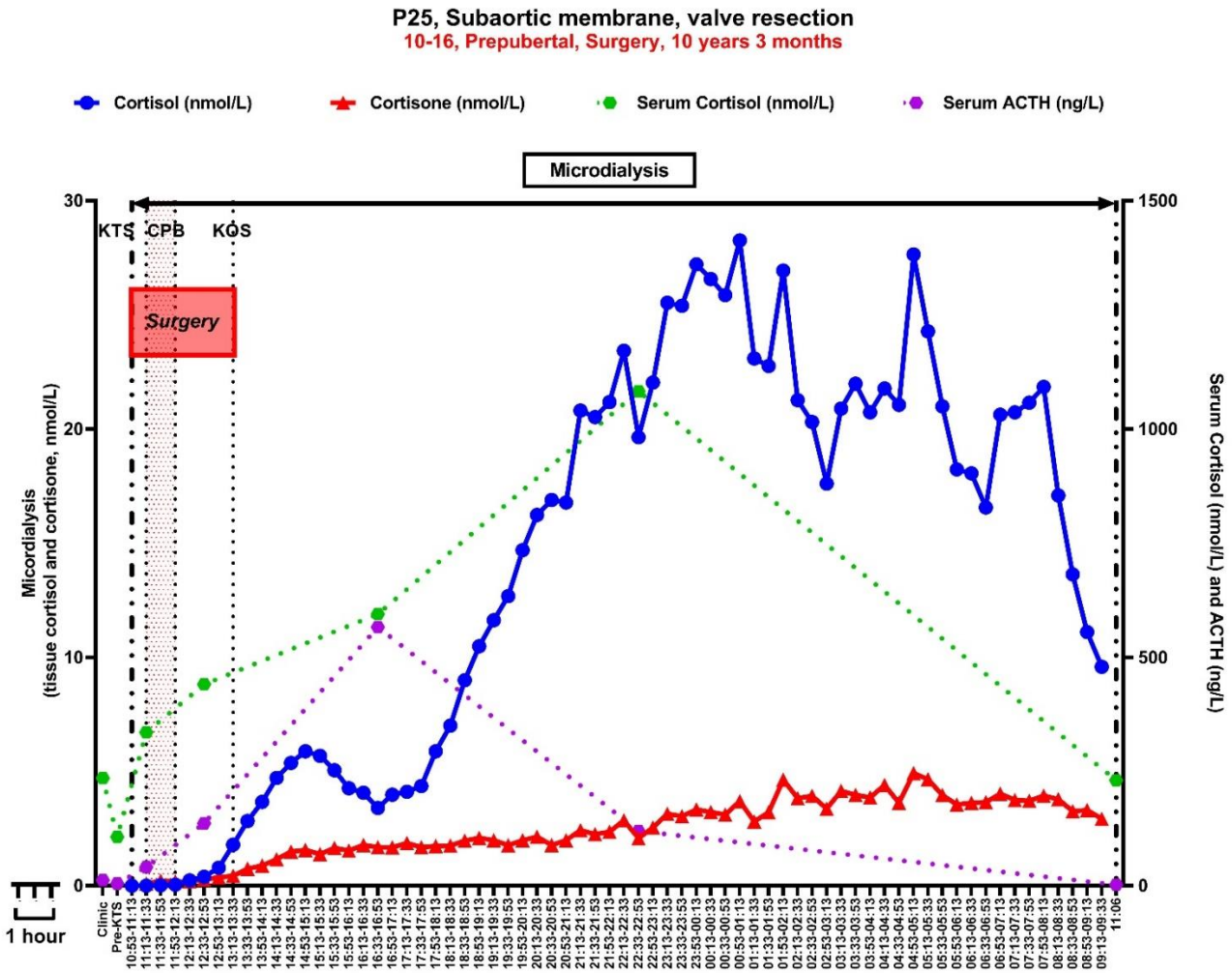

Figure 68 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

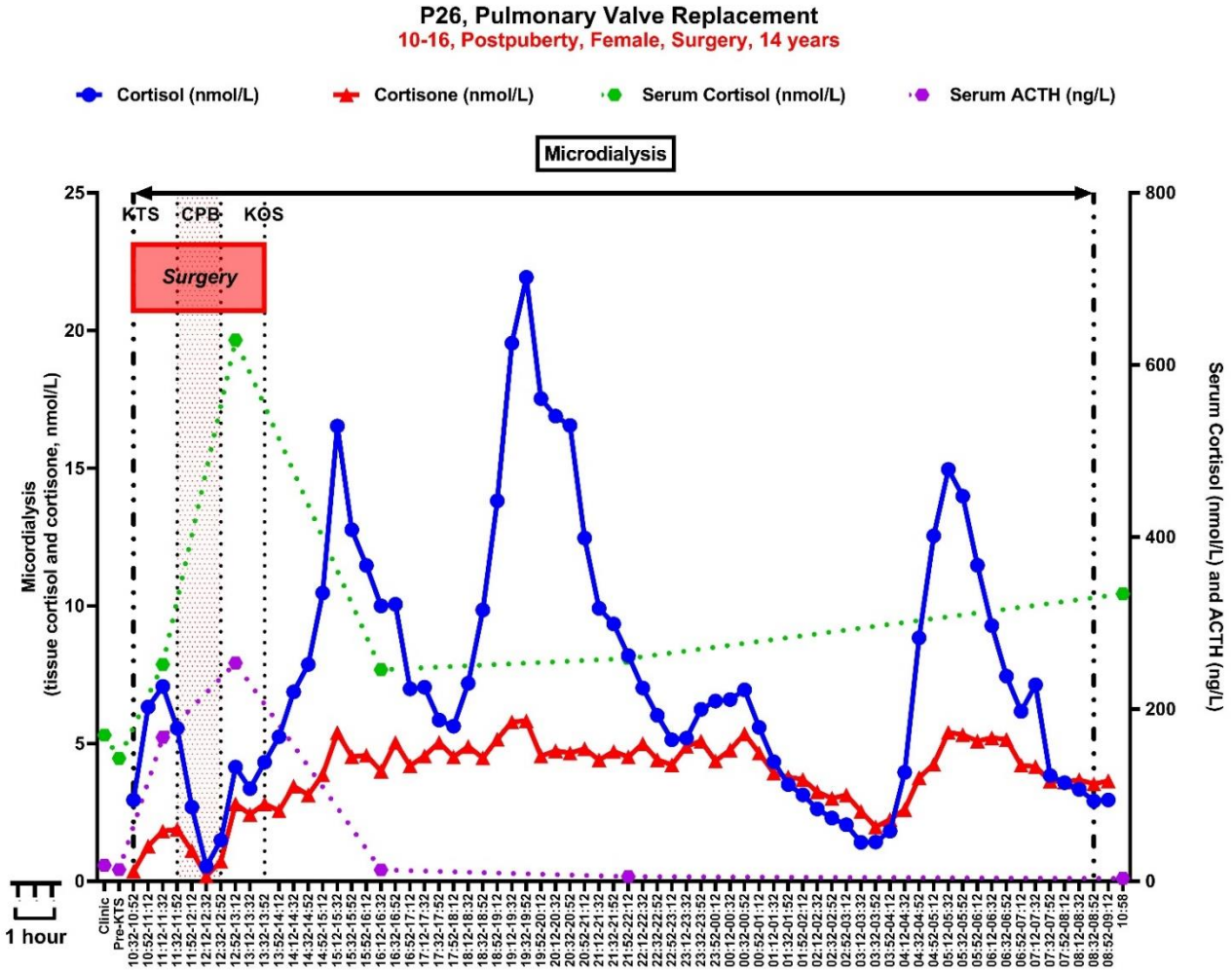

Figure 69 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

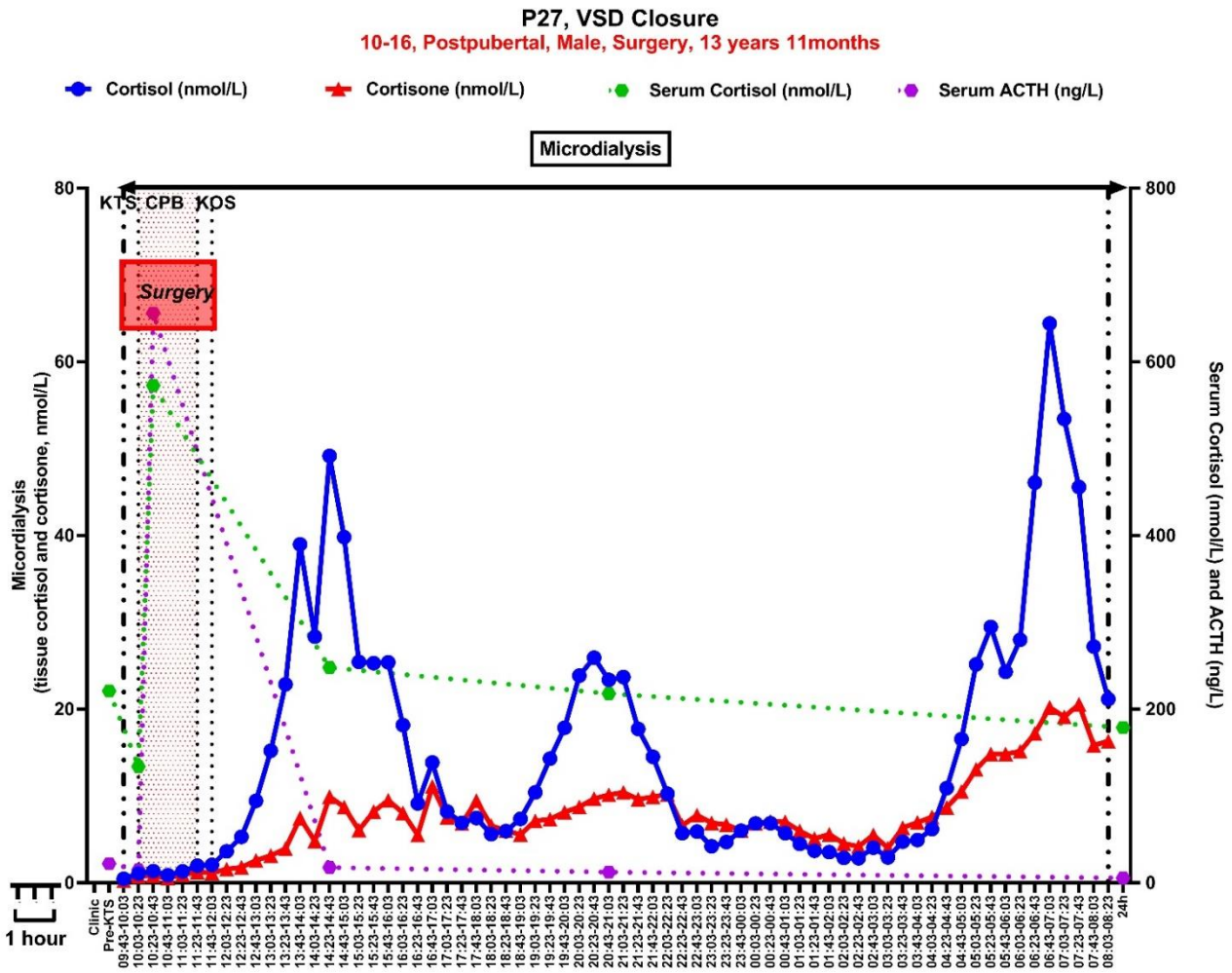

Figure 70 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

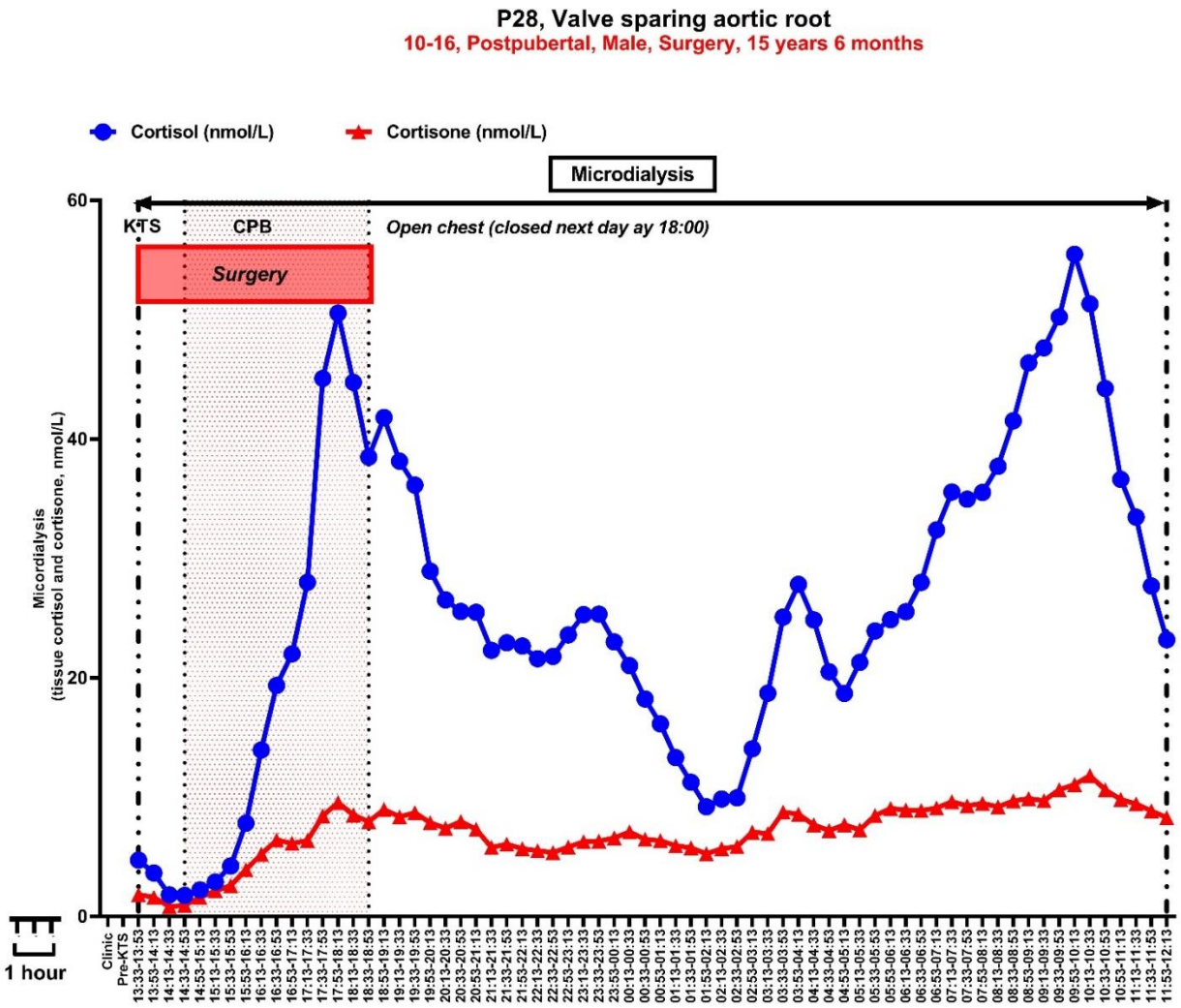

Figure 71 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

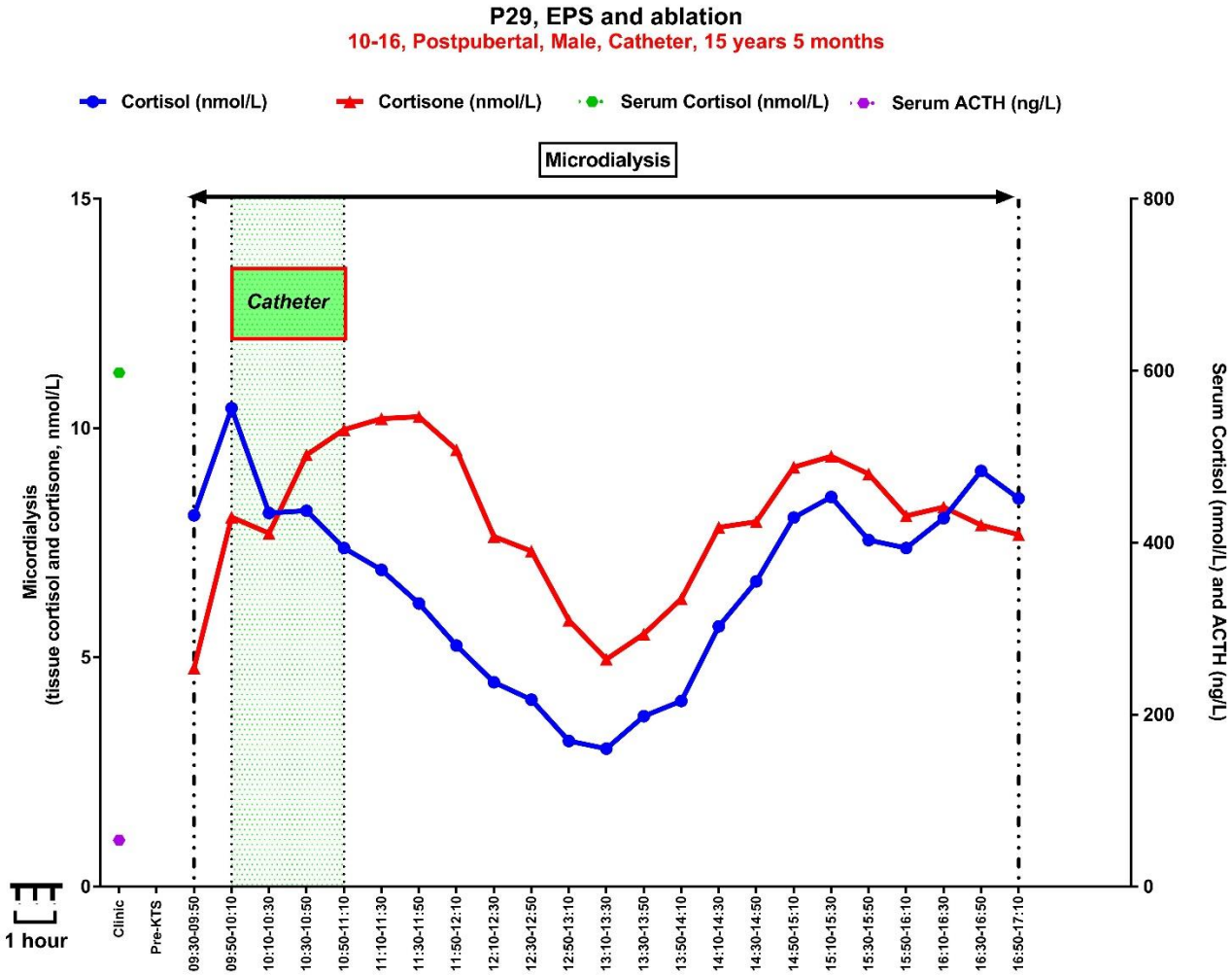

Figure 72 Cortisol and cortisone microdialysis profiles of a child undergoing a catheter procedure. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The green shaded box denotes the operative time (from KTS to KOS). The green dot denotes the serum cortisol level taken at the reference time point. The purple dot line denotes the ACTH serum concentration taken at the reference time point. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

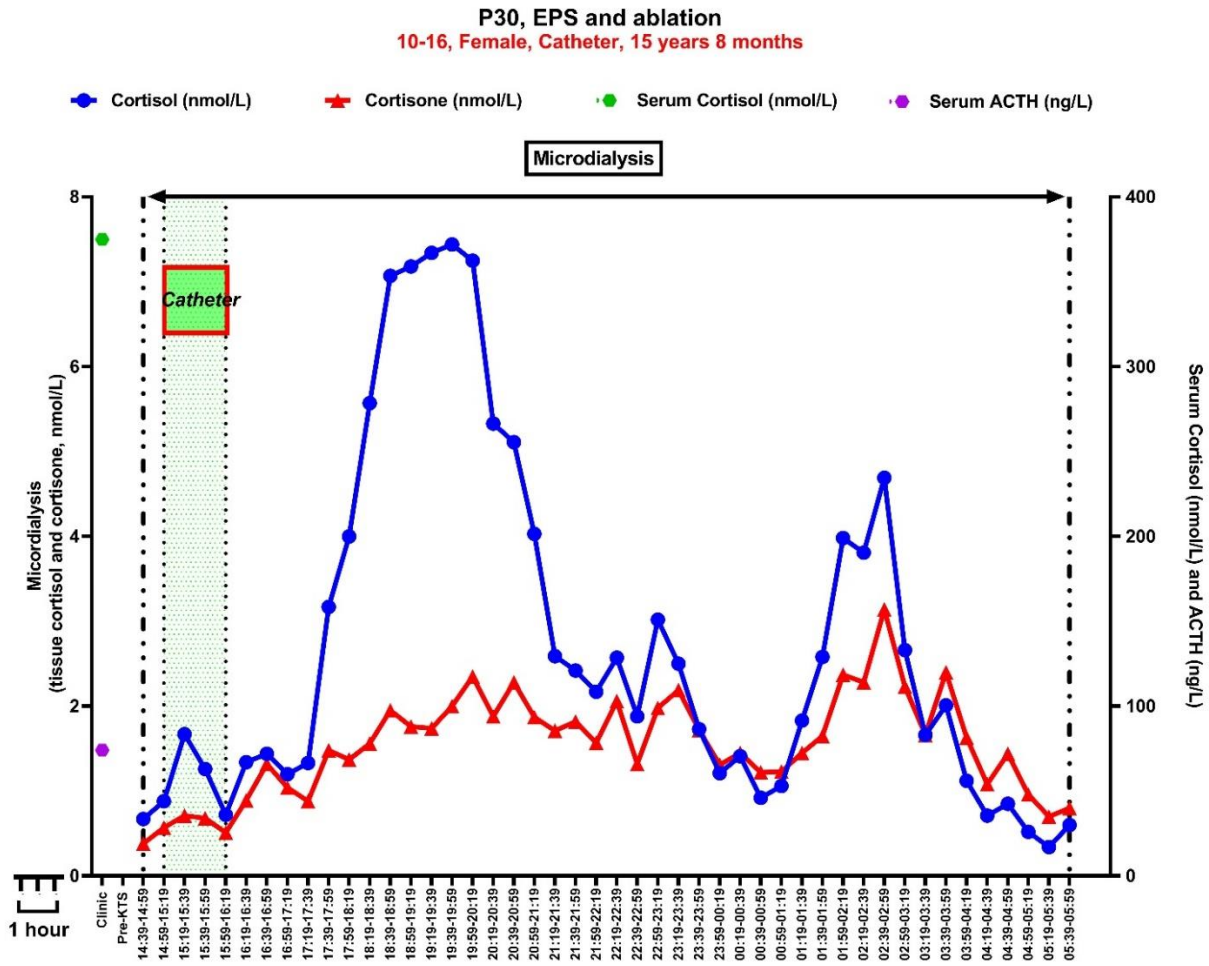

Figure 73 Cortisol and cortisone microdialysis profiles of a child undergoing a catheter procedure. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The green shaded box denotes the operative time (from KTS to KOS). The green dot denotes the serum cortisol level taken at the reference time point. The purple dot line denotes the ACTH serum concentration taken at the reference time point. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

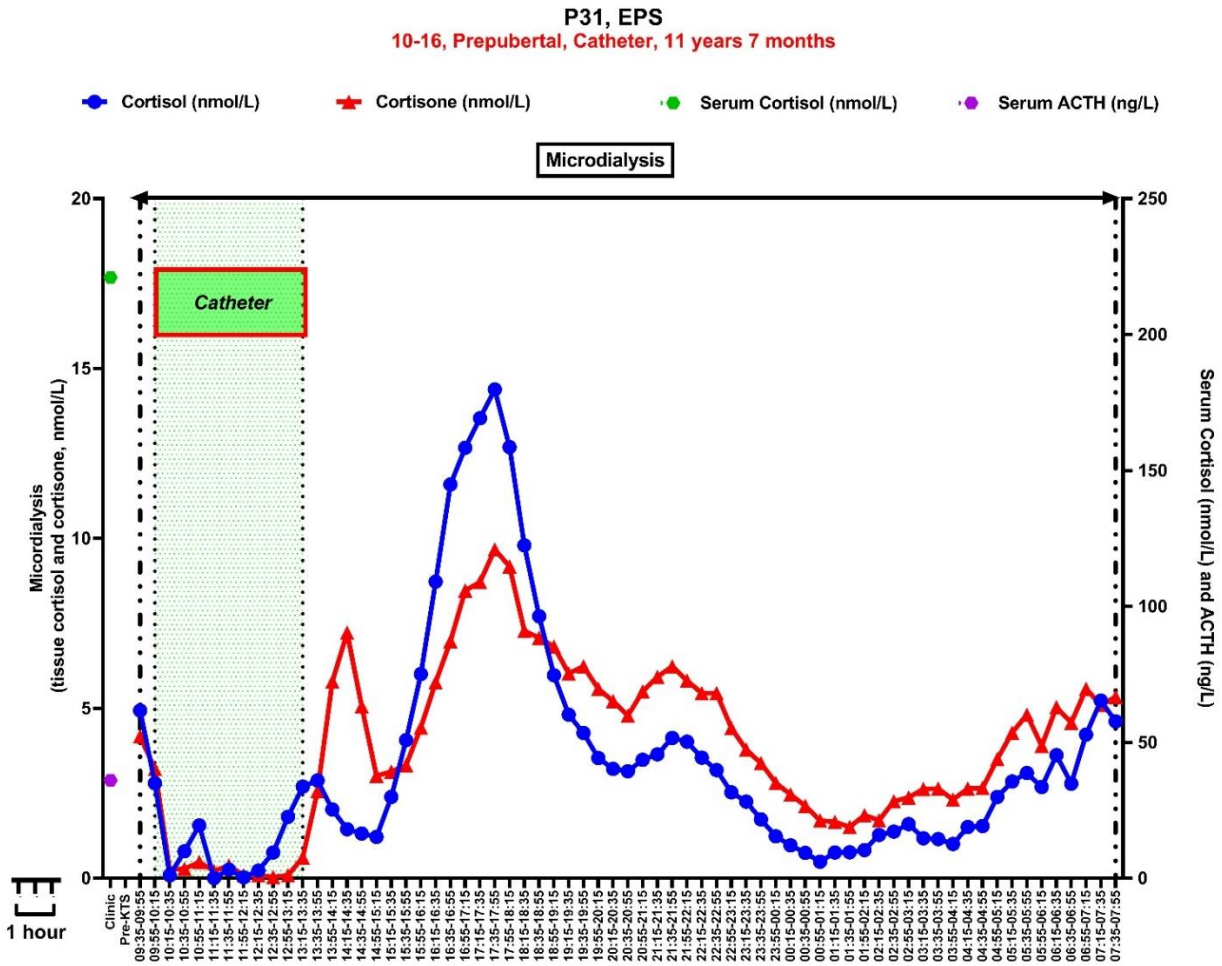

Figure 74 Cortisol and cortisone microdialysis profiles of a child undergoing a catheter procedure. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The green shaded box denotes the operative time (from KTS to KOS). The green dot denotes the serum cortisol level taken at the reference time point. The purple dot line denotes the ACTH serum concentration taken at the reference time point. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

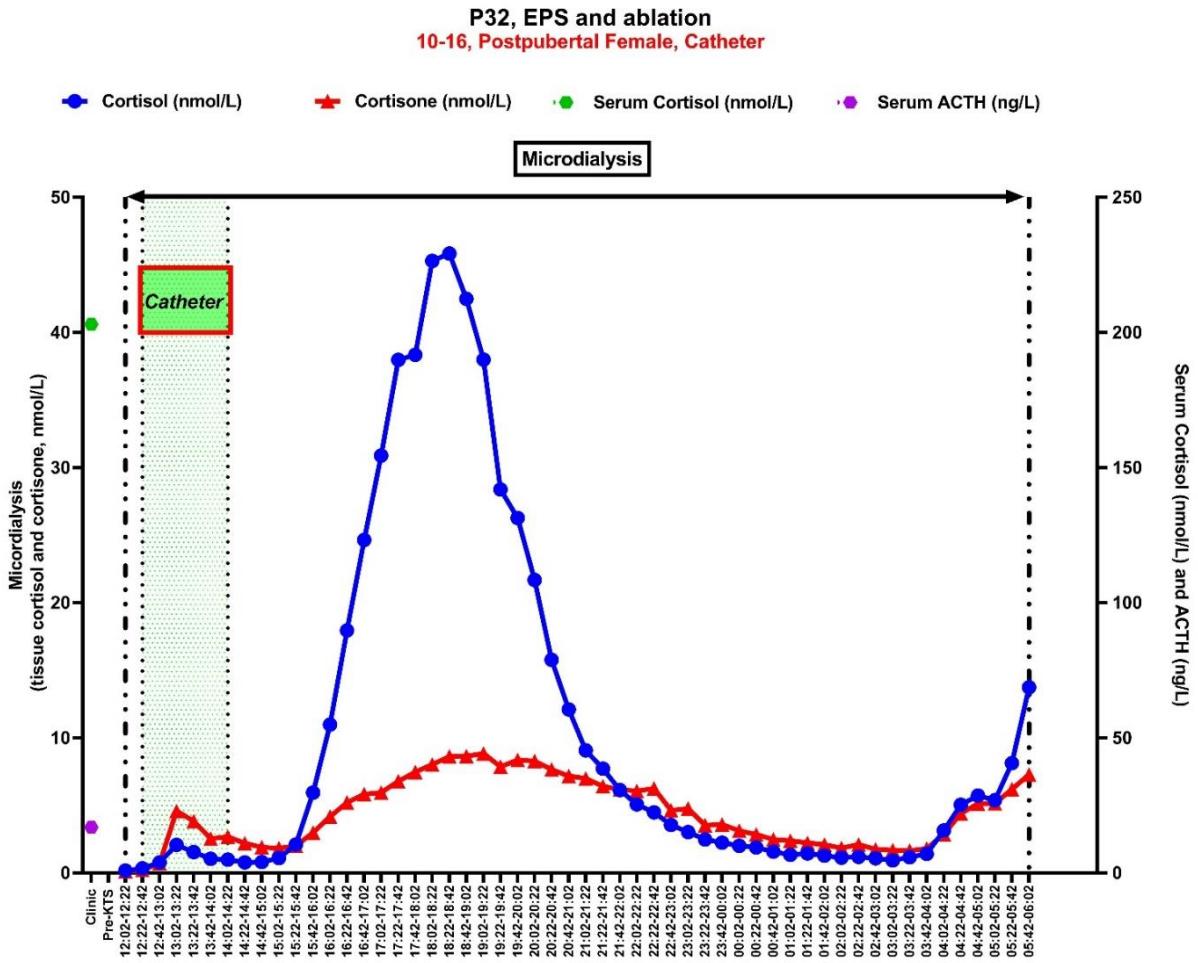

Figure 75 Cortisol and cortisone microdialysis profiles of a child undergoing a catheter procedure. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The green shaded box denotes the operative time (from KTS to KOS). The green dot denotes the serum cortisol level taken at the reference time point. The purple dot line denotes the ACTH serum concentration taken at the reference time point. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

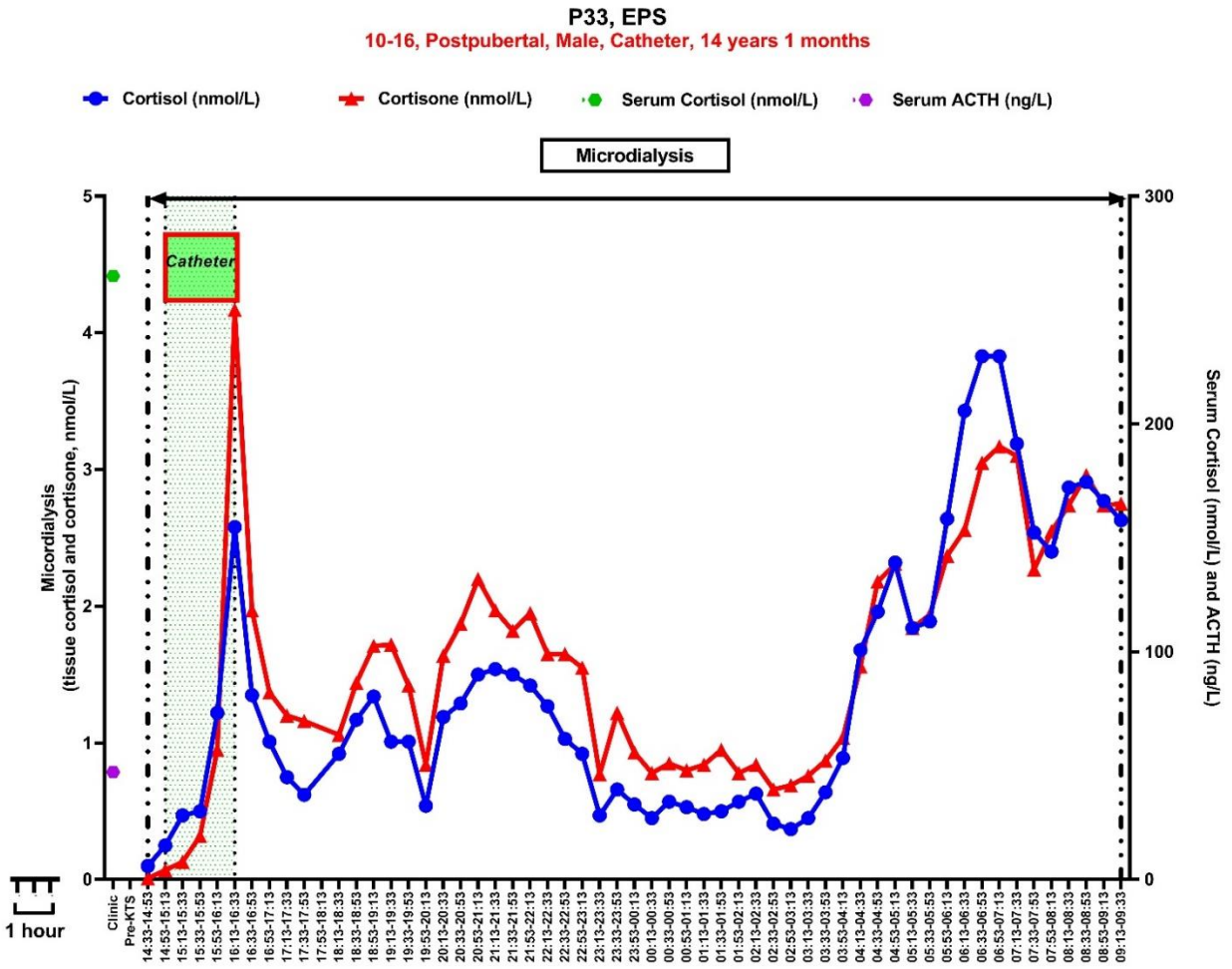

Figure 76 Cortisol and cortisone microdialysis profiles of a child undergoing a catheter procedure. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The green shaded box denotes the operative time (from KTS to KOS). The green dot denotes the serum cortisol level taken at the reference time point. The purple dot line denotes the ACTH serum concentration taken at the reference time point. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

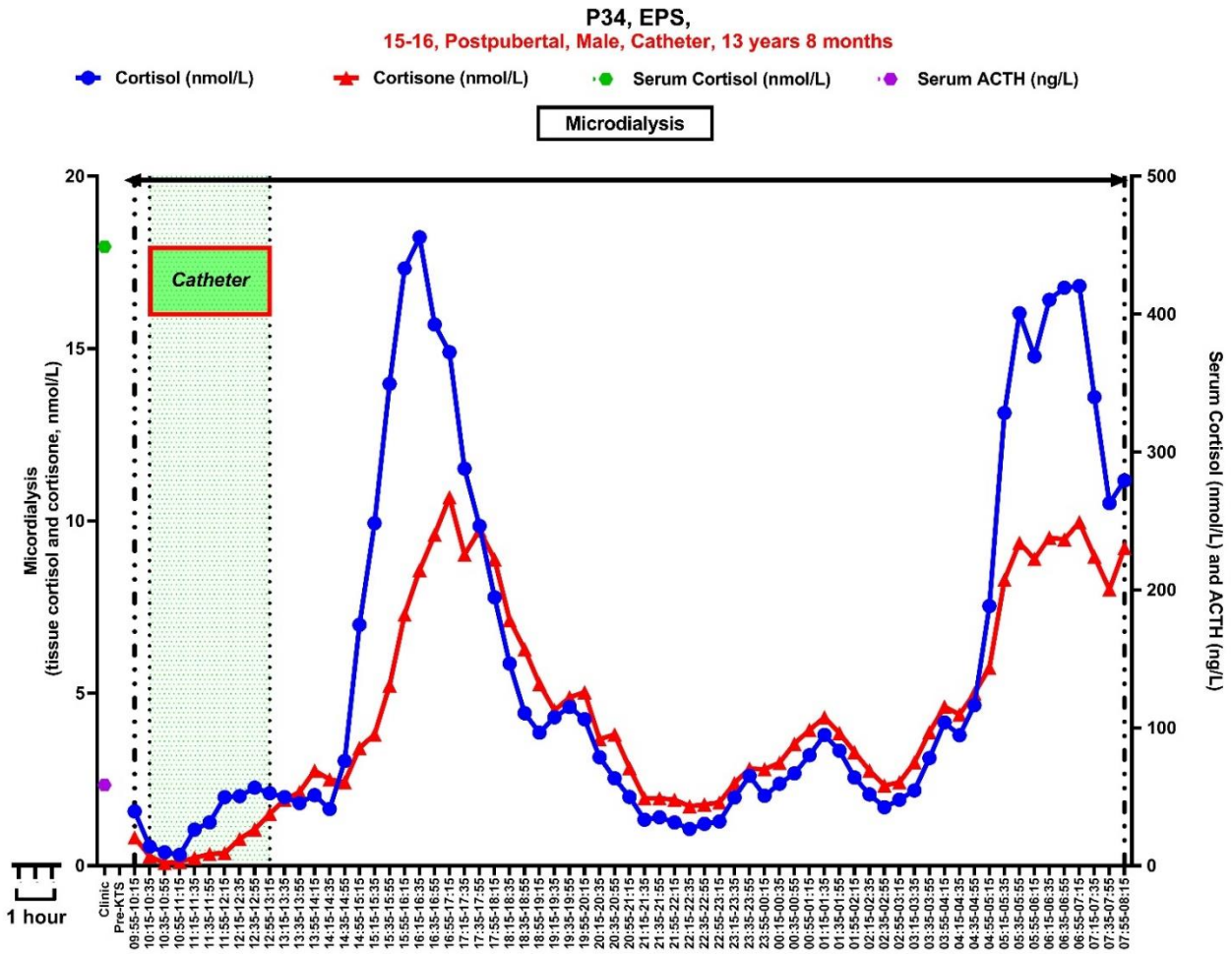

Figure 77 Cortisol and cortisone microdialysis profiles of a child undergoing a catheter procedure. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The green shaded box denotes the operative time (from KTS to KOS). The green dot denotes the serum cortisol level taken at the reference time point. The purple dot line denotes the ACTH serum concentration taken at the reference time point. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

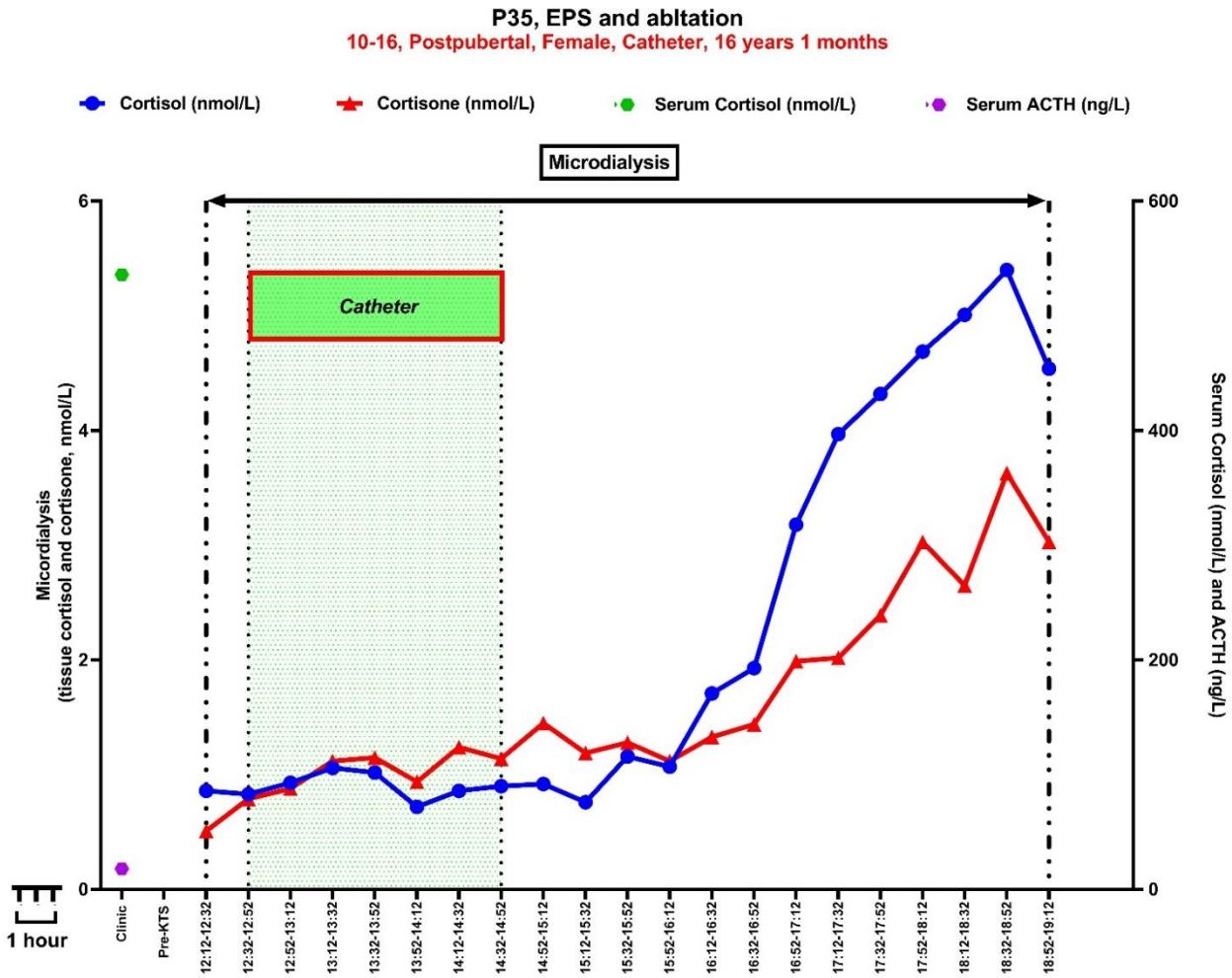

Figure 78 Cortisol and cortisone microdialysis profiles of a child undergoing a catheter procedure. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The green shaded box denotes the operative time (from KTS to KOS). The green dot denotes the serum cortisol level taken at the reference time point. The purple dot line denotes the ACTH serum concentration taken at the reference time point. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

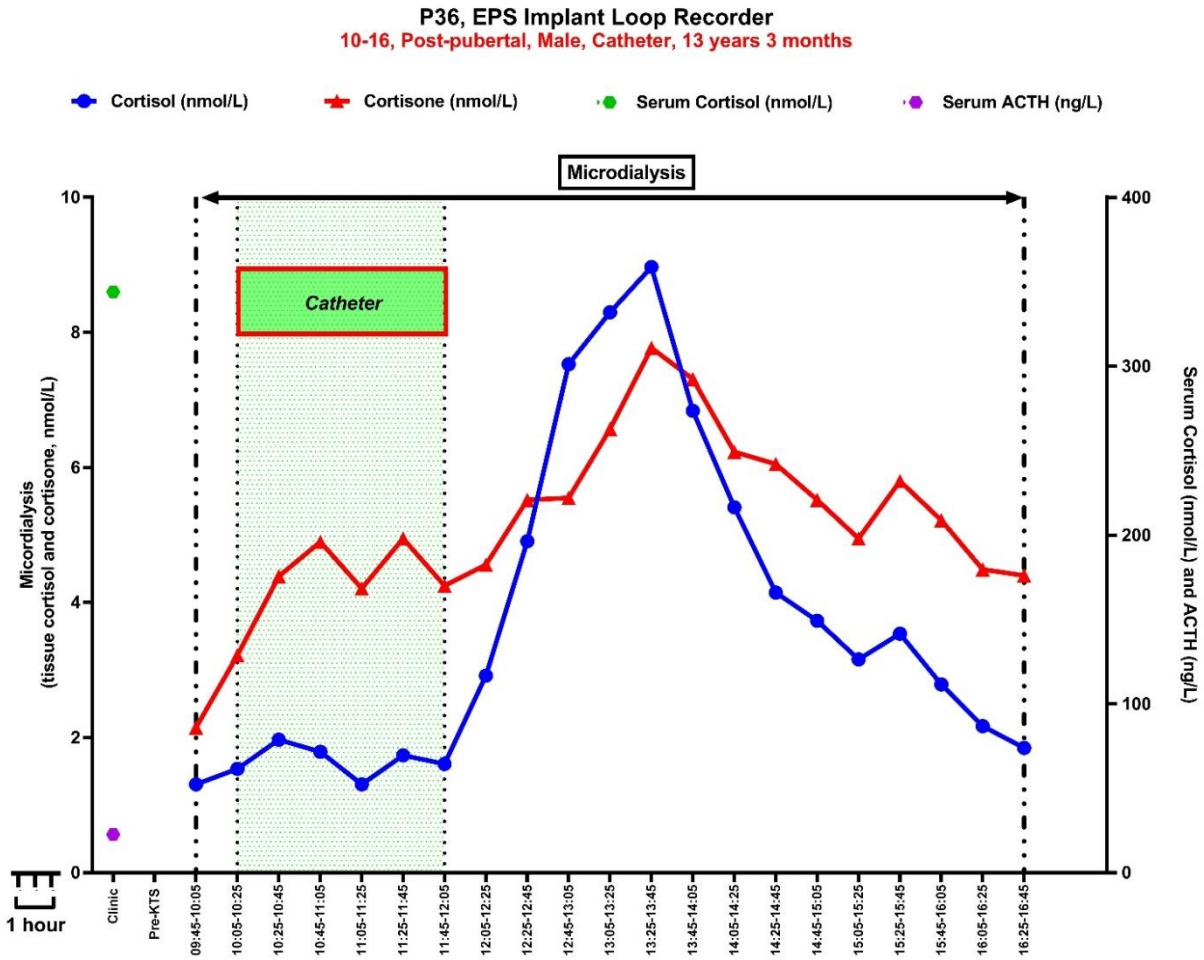

Figure 79 Cortisol and cortisone microdialysis profiles of a child undergoing a catheter procedure. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The green shaded box denotes the operative time (from KTS to KOS). The green dot denotes the serum cortisol level taken at the reference time point. The purple dot line denotes the ACTH serum concentration taken at the reference time point. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

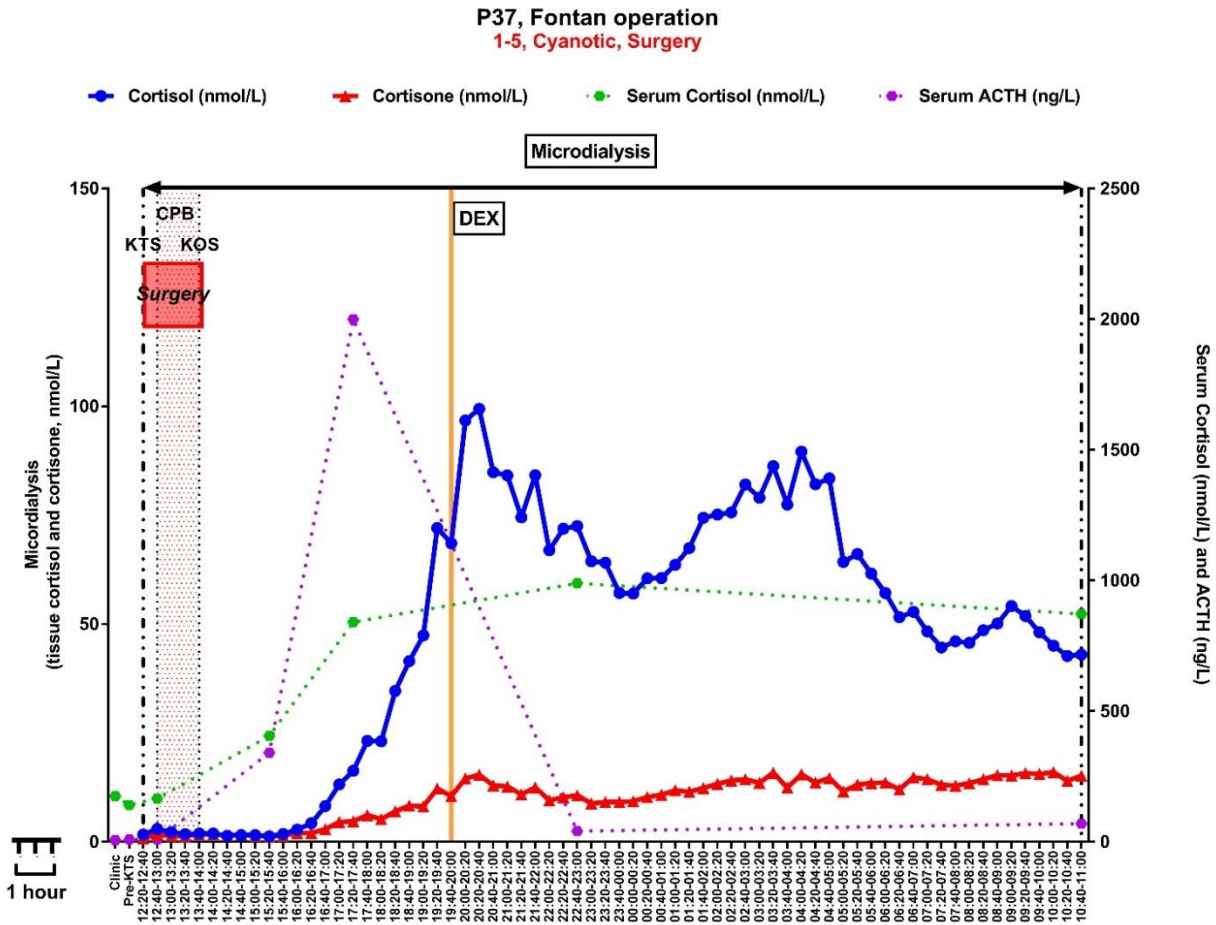

Figure 80 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery who received intravenous Dexamethasone during sampling. The yellow line marks the time of intravenous administration. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

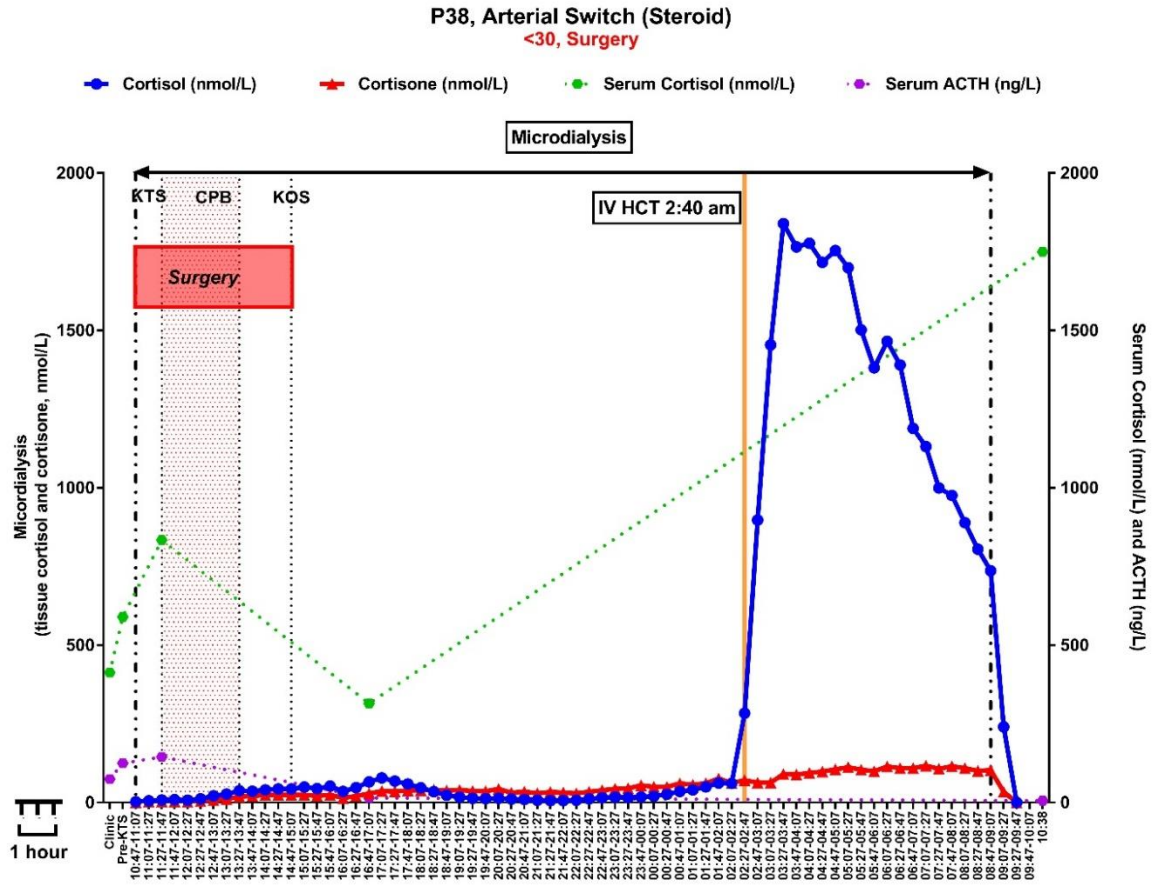

Figure 81 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery who received intravenous Hydrocortisone during sampling. The yellow line marks the time of intravenous hydrocortisone administration. The cortisol profile after IV glucocorticoid administration is depicted in the next graph (for better understanding) Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

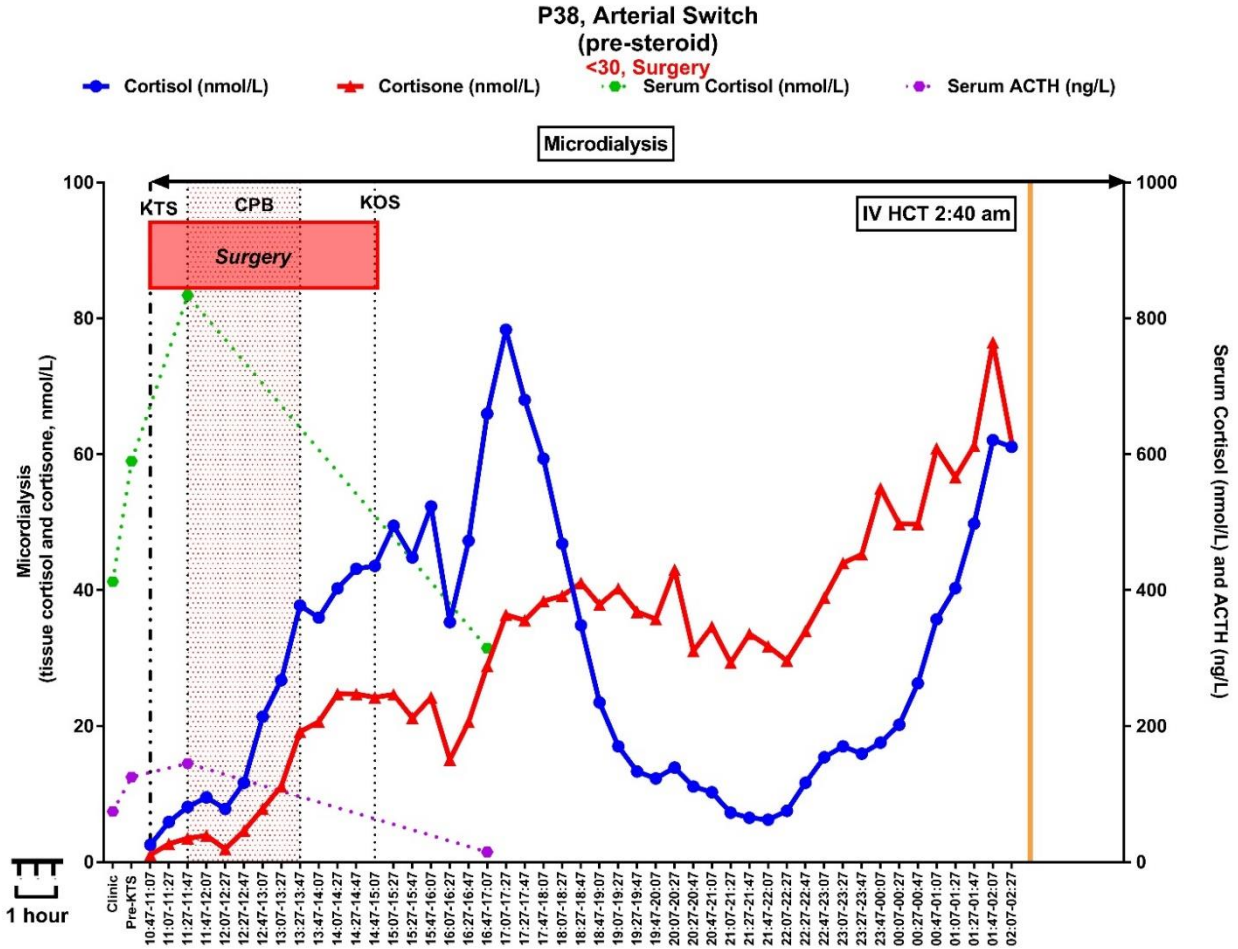

Figure 82 Cortisol and cortisone microdialysis profiles of a child undergoing heart surgery who received intravenous Hydrocortisone during sampling. The yellow line marks the time of intravenous hydrocortisone administration. Abbreviations: KTS: knife to skin; CPB: cardiopulmonary by-pass time (red dotted area). KOS – knife of skin time – end of the operation. The red shaded box denotes the operative time (from KTS to KOS). In dotted green lines are illustrated the serum cortisol levels taken at the reference time points (seven). The purple dotted line denotes the ACTH serum concentration taken at the reference time points (seven) is illustrated. On the right Y-axis, the serum cortisol/ACTH concentration is depicted. The blue line denotes the tissue cortisol and the red line the tissue cortisone levels taken every 20 minutes using the automated microdialysis system. Each point on the X-axis represents one tissue cortisol sample collected over a 20-minute interval. The two black dotted lines mark the microdialysis sampling period.

11.6.4 Overview of Cortisol/Cortisone changes by age group

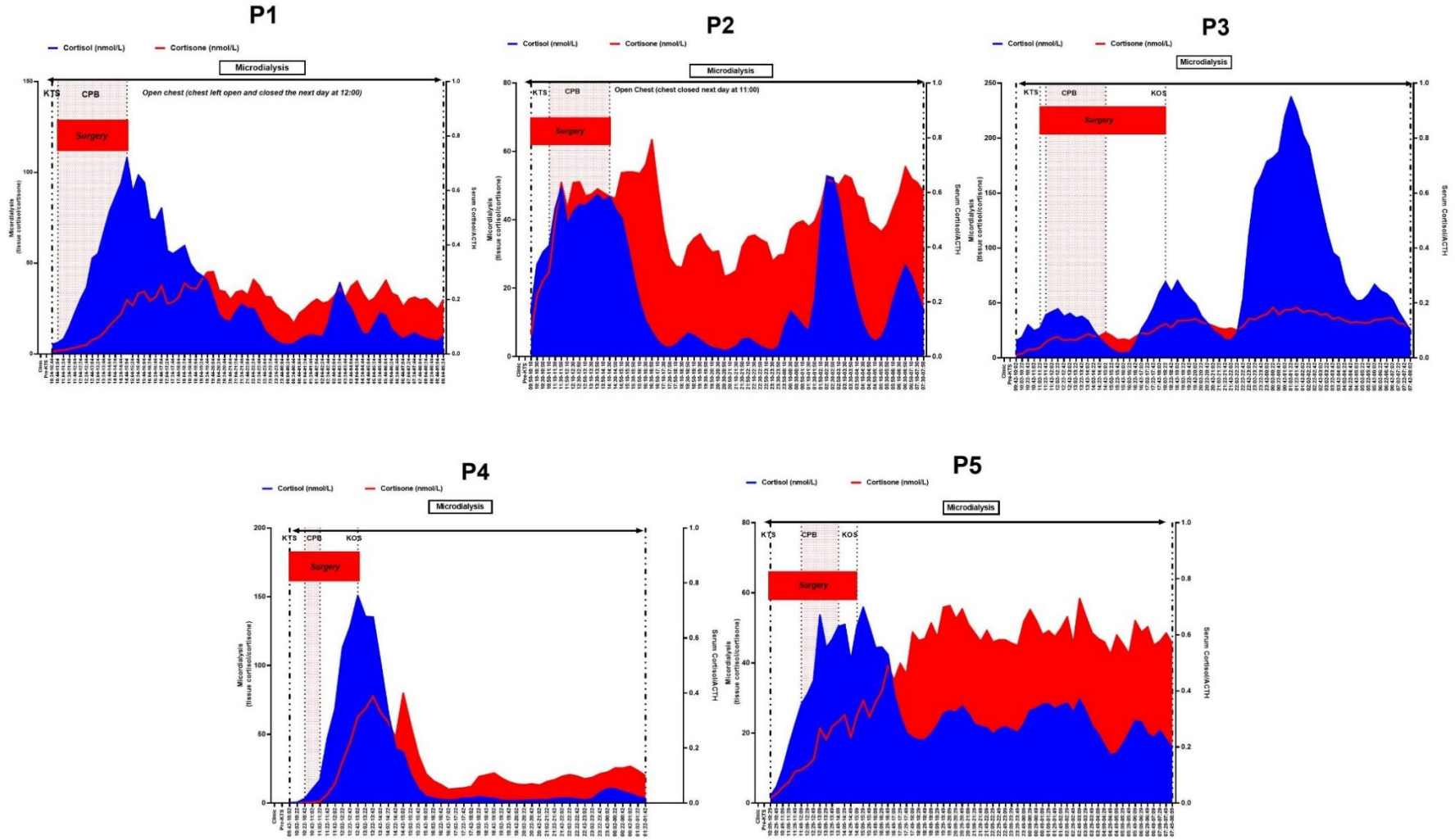

Figure 83 Cortisol and cortisone profiles of neonates undergoing surgery. The cortisol area is marked in blue. The cortisone response that overlaps the cortisol response is marked in red.

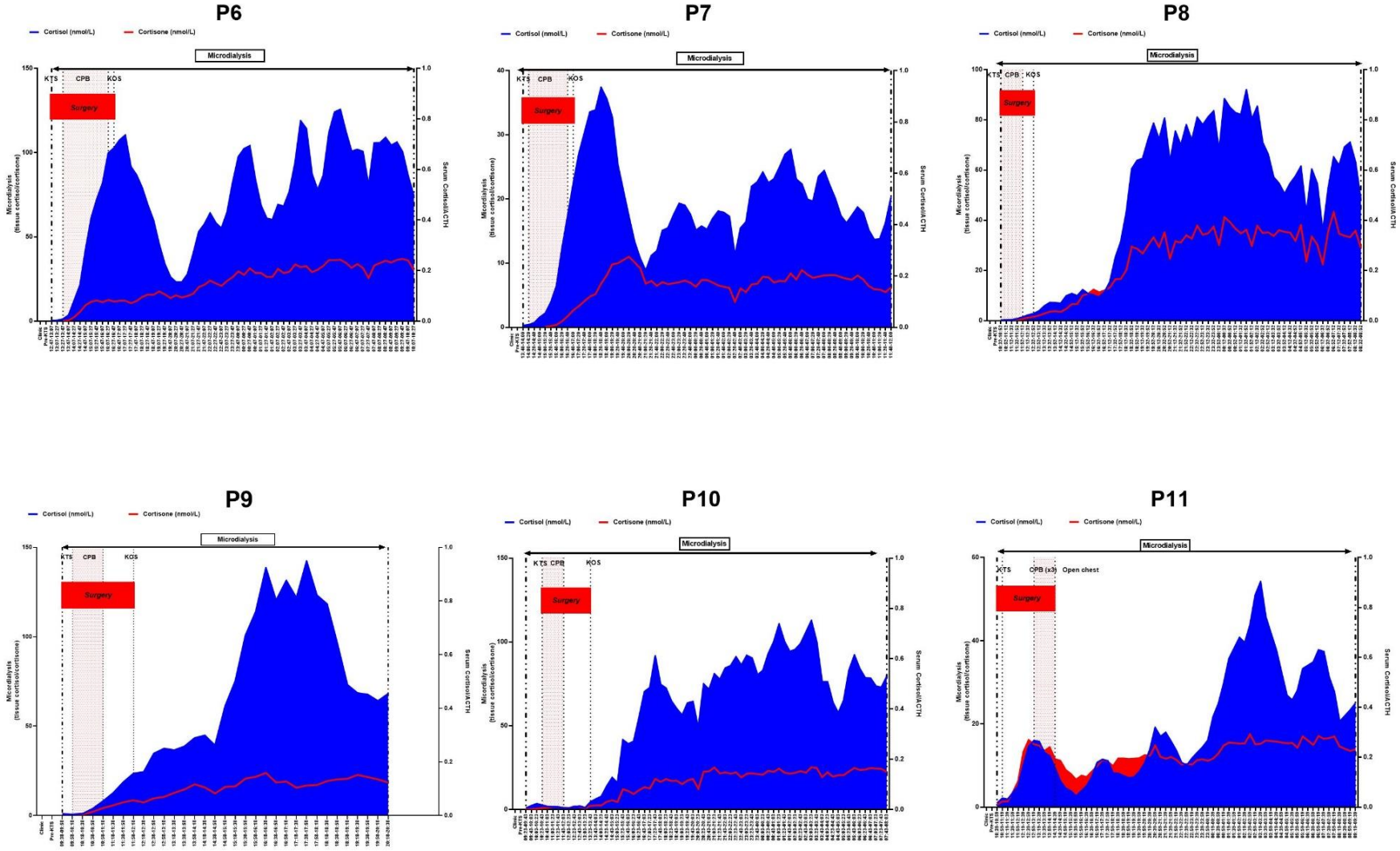

Figure 84 Cortisol and cortisone profiles of children aged >30 days-1 year undergoing surgery. The cortisol area is marked in blue. The cortisone response that overlaps the cortisol response is marked in red.

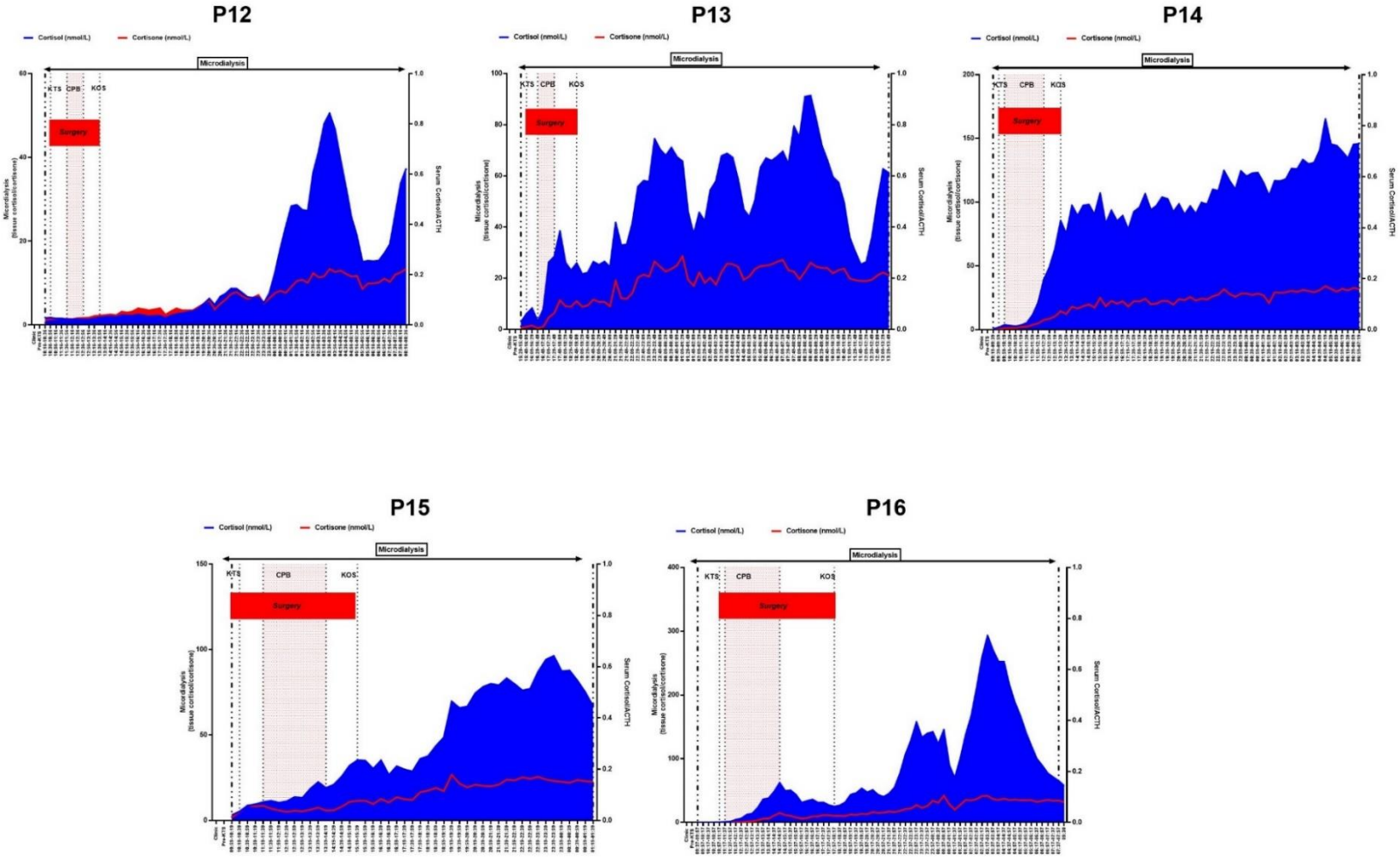

Figure 85 Cortisol and cortisone profiles of children aged >30 days-1 year undergoing surgery (continued). The cortisol area is marked in blue. The cortisone response that overlaps the cortisol response is marked in red.

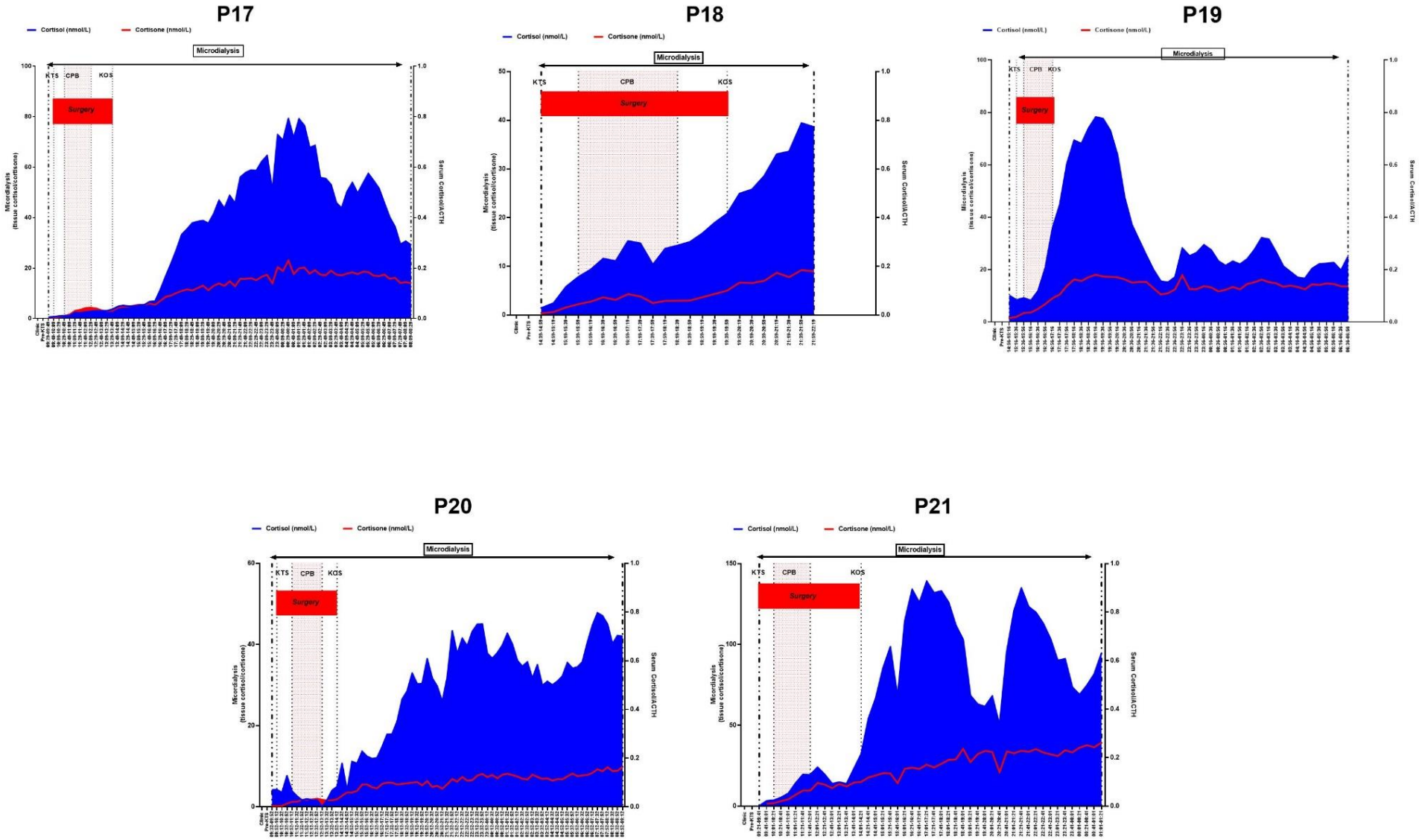

Figure 86 Cortisol and cortisone profiles of children aged 1-5 year undergoing surgery. The cortisol area is marked in blue. The cortisone response that overlaps the cortisol response is marked in red.

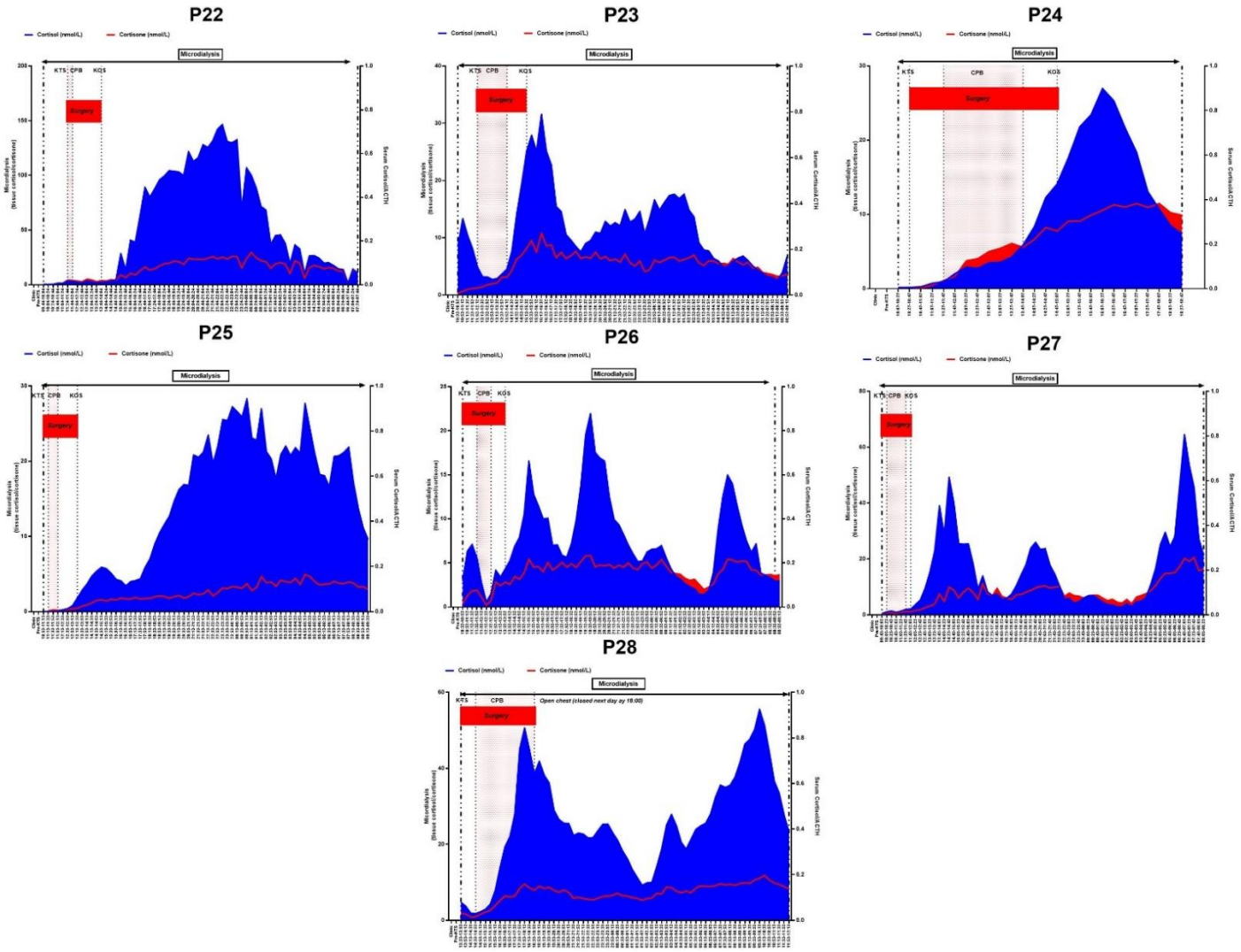

Figure 87 Cortisol and cortisone profiles of children aged 10-16 year undergoing surgery. The cortisol area is marked in blue. The cortisone response that overlaps the cortisol response is marked in red.

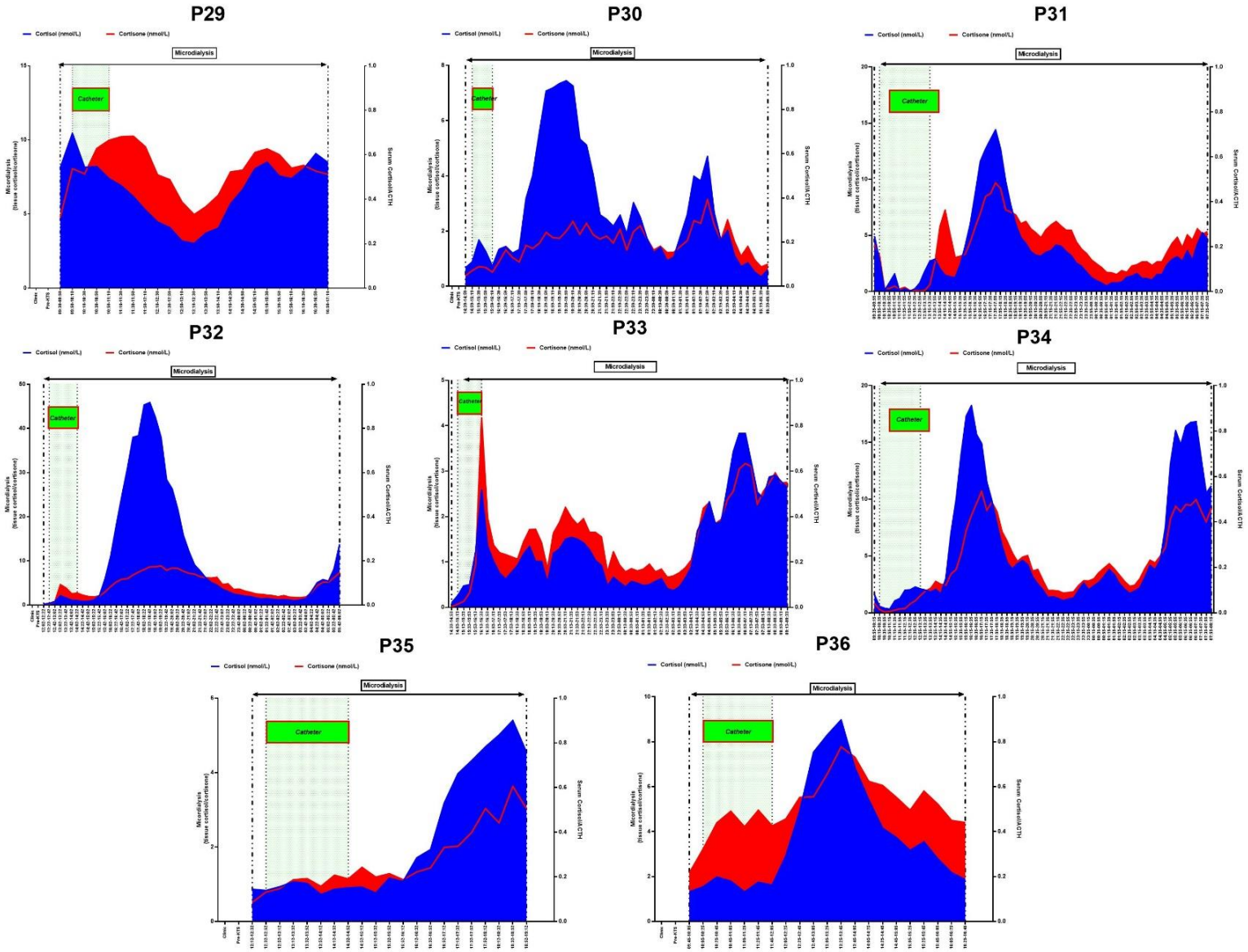

Figure 88 Cortisol and cortisone profiles of children aged 10-16 year undergoing catheter procedures. The cortisol area is marked in blue. The cortisone response that overlaps the cortisol response is marked in red.

Patient	Recruitment group	Procedure	AUC Cortisol nmol/L*20 min	AUC Cortisone nmol/L*20 min	Cortisol: Cortisone
P1	<30 days	Surgery	2431	2599	0.935359754
P2	<30 days	Surgery	1649	3819	0.431788426
P3	<30 days	Surgery	4538	1871	2.425440941
P4	<30 days	Surgery	1197	1175	1.018723404
P5	<30 days	Surgery	1013	960.3	1.054878684
P6	30 days -1 year	Surgery	4741	1444	3.283240997
P7	30 days -1 year	Surgery	1567	548.3	2.857924494
P8	30 days -1 year	Surgery	3147	1579	1.993033566
P9	30 days -1 year	Surgery	2017	448.6	4.496210432
P10	30 days -1 year	Surgery	978.2	247.1	3.958721166
P11	30 days -1 year	Surgery	286.5	327.6	0.874542125
P12	30 days -1 year	Surgery	78.66	94.6	0.831501057
P13	30 days -1 year	Surgery	1186	435.6	2.722681359
P14	30 days -1 year	Surgery	2114	453.5	4.661521499
P15	30 days -1 year	Surgery	970.1	368.8	2.630422993
P16	30 days -1 year	Surgery	5605.4	1275.6	4.39432424
P17	1-5 years	Surgery	2412	810.8	2.974839665
P18	1-5 years	Surgery	158.4	39.44	4.016227181
P19	1-5 years	Surgery	1134	396.2	2.862190813
P20	1-5 years	Surgery	2501	533.7	4.68615327
P21	1-5 years	Surgery	1933	571.5	3.382327209
P22	10-16 years	Surgery	1373	313.5	4.379585327
P23	10-16 years	Surgery	398	173.1	2.299248989
P24	10-16 years	Surgery	252.8	166.1	1.521974714
P25	10-16 years	Surgery	213.2	43.75	4.873142857
P26	10-16 years	Surgery	294.6	117.3	2.511508951
P 27	10-16 years	Surgery	1031	521	1.978886756
P28	10-16 years	Surgery	1725	480.6	3.589263421
P29	10-16 years	Catheter	144.3	176.5	0.817563739
P30	10-16 years	Catheter	124.2	70	1.774285714
P31	10-16 years	Catheter	225.3	259.1	0.869548437
P32	10-16 years	Catheter	549.2	236.7	2.320236586
P33	10-16 years	Catheter	76.81	88.44	0.868498417
P34	10-16 years	Catheter	371.6	289.1	1.285368385
P35	10-16 years	Catheter	43.14	32.55	1.325345622
P36	10-16 years	Catheter	75.96	104.7	0.725501433

Table 13 Total AUC for cortisol and cortisone in all patients (N=36)

11.6.5 Grouped cortisol analysis

All cortisol profiles were pulsatile. There was no patient that had a suppressed endogenous cortisol response. The tissue cortisol levels increased significantly at a variable interval from the procedure. The majority of the surgical patient had up to 100-fold increase or more in their cortisol levels from basal while the cortisol peaks in the catheter patients were less pronounced (10-20-fold increase). Table 13 summarises the total AUC for all patients by recruitment group and age.

11.6.6 Changes in AUC for cortisol and cortisone by age

There was a significant effect of age of children undergoing cardiac procedures on the changes in the AUC for cortisol (P=0.0045), AUC for cortisone and in the ratio of AUC for cortisol to AUC cortisone (P=0.0097) (Figure 89). In the post hoc analysis, the AUC for cortisol in neonates was significantly larger than the 10-16 years patients (P=0.0363). Similarly, the AUC for cortisol in infants (age 1-5) was significantly larger than the 10-16 years (P=0.0242) (Figure 89 A). The AUC for cortisone was significantly larger in neonates compared to 10-16 years group (P=0.0003), and there was a trend towards a larger area in infants compared to 10-16 years older patients (P=0.0539) (Figure 89 B). The ratio of the AUC for cortisol to cortisone was significantly lower in neonates compared to 1-5 years old children (P=0.0214) (Figure 89 C).

11.6.7 Changes in AUC for cortisol and cortisone by type of procedure

The AUC for cortisol (Figure 90 A) and cortisone (Figure 90 B) were significantly larger (P<0.0001 and P=0.0017, respectively) in the patients undergoing surgery compared to the patients undergoing catheter procedures. The ratio of cortisol to cortisone ratio was significantly lower in catheter patients (P=0.0023) (Figure 90 C).

11.6.8 Procedure specific changes in AUC

I cannot make any solid statements regarding the procedure specific changes as the numbers within each procedure were too small. However, the increases in AUC for cortisol were broadly consistent with the complexity of the operation (Figure 91). For example, Norwood procedures[4] followed by arterial switch procedures had the highest recorded AUC. This fitted well with RACHS scoring. In contrast, catheter procedures had the lowest AUC for cortisol.

11.6.9 Changes in AUC for cortisol and cortisone by type of underlying heart defect.

We found no significant difference in the AUC for cortisol (P=0.3791) (Figure 92, A), for cortisone (P=0.1281) (Figure 92, B) and the ratios of AUC for cortisol to AUC for cortisone (P=0.9604) (Figure 92, B) in patients with a acyanotic heart defects (pre-op oxygen saturation > 89%) versus cyanotic heart defect (pre-op oxygen saturation ≤ 89%).

[4] The Norwood procedure is probably one of the most complex operations in congenital heart surgery and in heart surgery in general. It is the first surgery out of three staged heart operations that aims to create new systemic circulation (univentricular) in patients born with hypoplastic left heart syndrome.

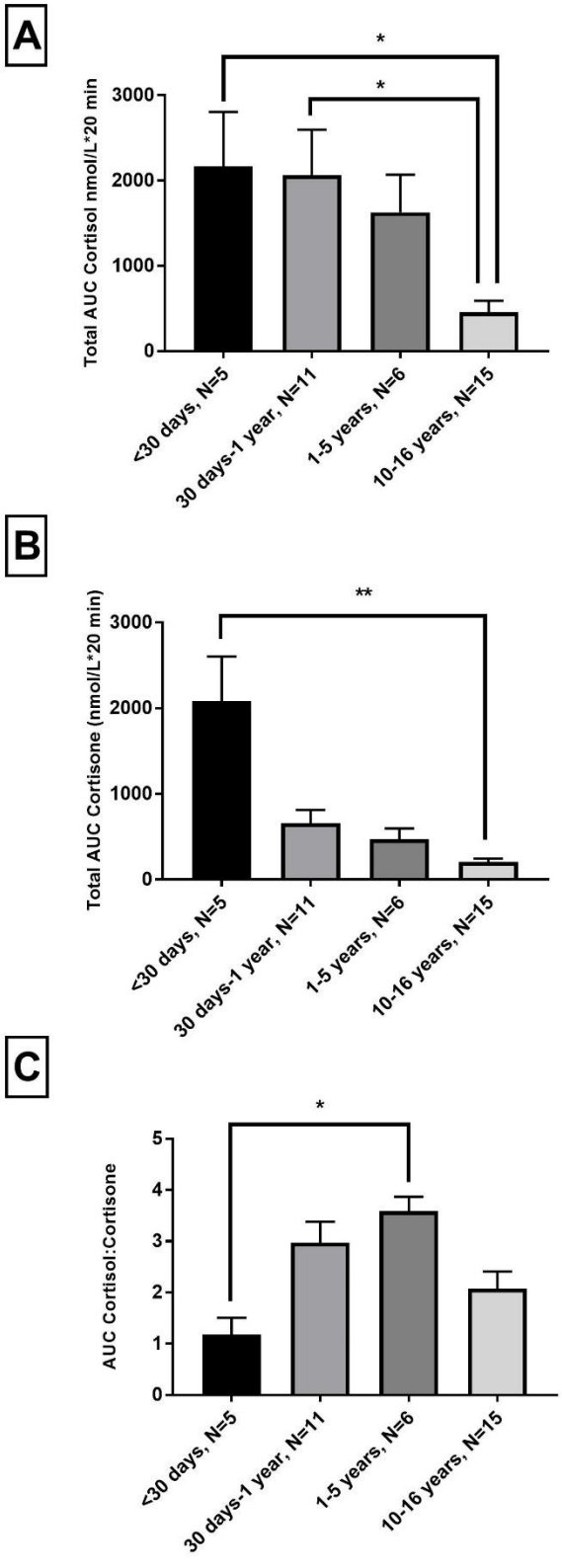

*Figure 89 Changes in AUC cortisol/cortisone by age group. Data are mean of AUC per age group ±SEM; data were analysed by one-way ANOVA followed by Kruskal-Wallis post-hoc-test; * P<0.05,**<P0.005; A - AUC for cortisol, B- AUC for cortisone, C- the ratio of AUC cortisol/cortisone.*

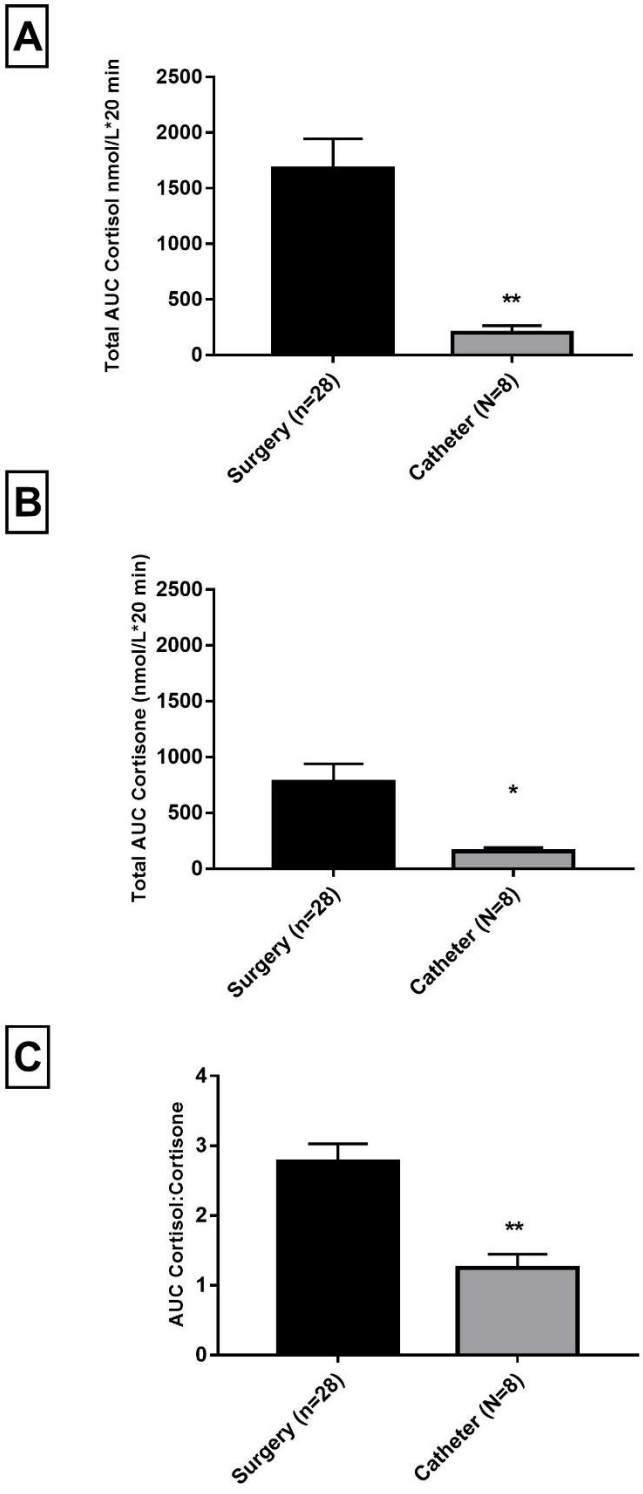

*Figure 90 Changes in AUC cortisol/cortisone according to type procedure (surgery vs catheter). Data are mean of AUC per procedure group ±SEM; data were analysed using the Mann-Whitney test; * P<0.05, **<P0.005; A - AUC for cortisol, B- AUC for cortisone, C- ratio of AUC cortisol/cortisone.*

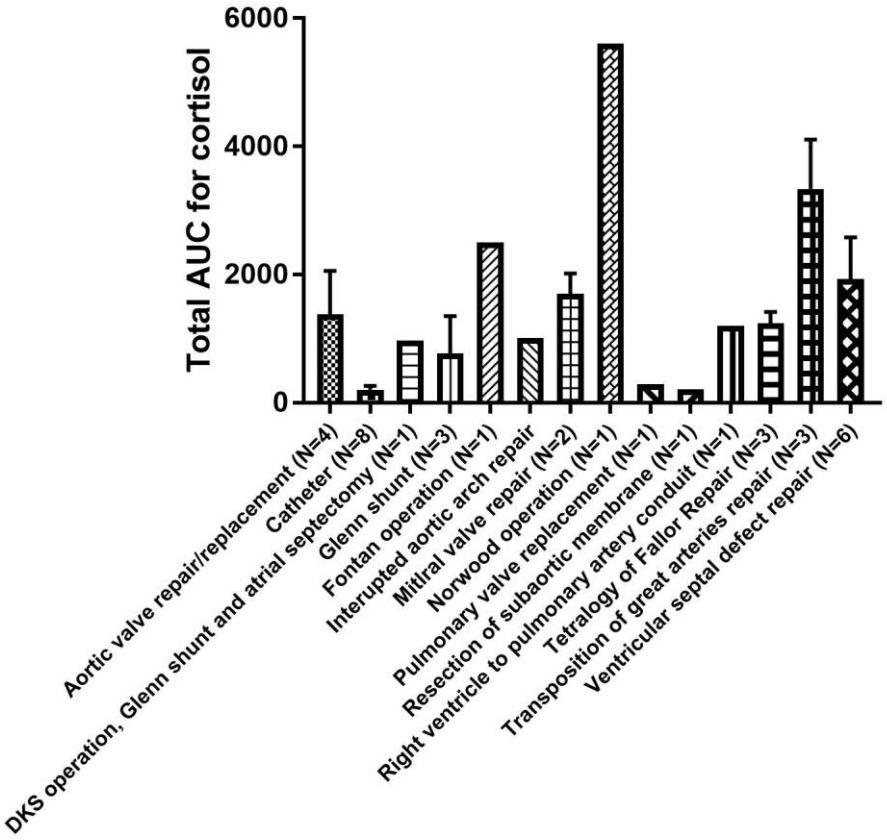

Figure 91 Procedure specific changes of AUC for cortisol

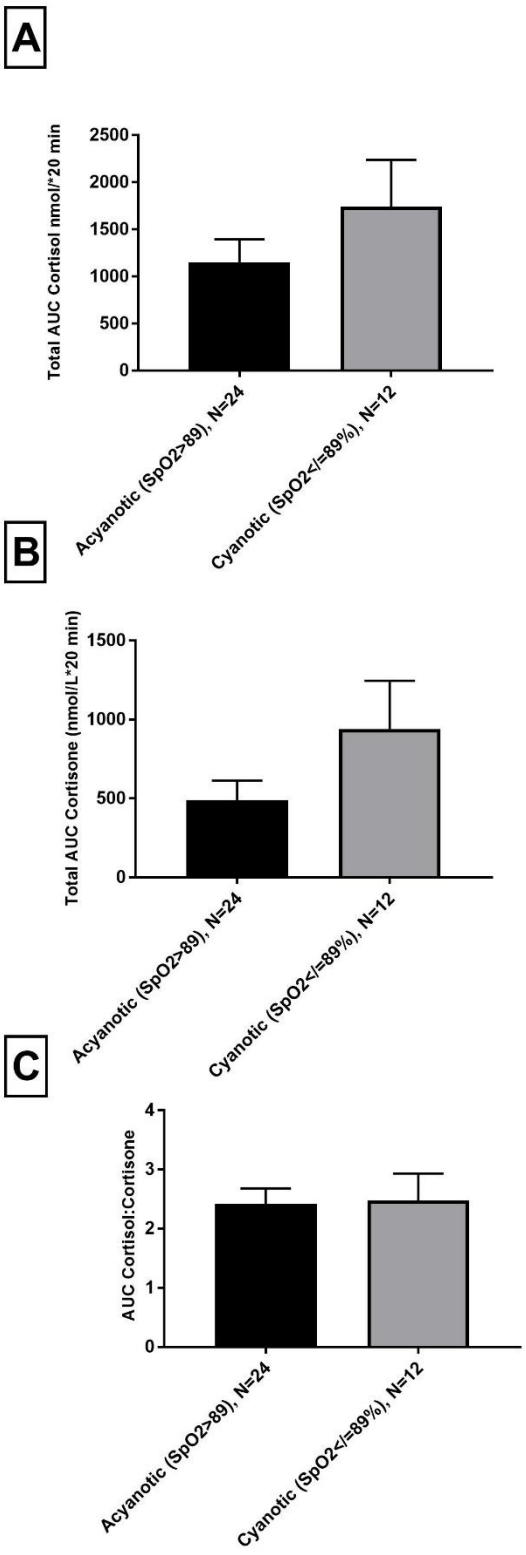

Figure 92 Changes in AUC cortisol/cortisone according to underlying congenital heart defect (acyanotic vs cyanotic). Data are mean of AUC per procedure group ±SEM; data were analysed using the Mann-Whitney test; A - AUC for cortisol, B- AUC for cortisone, C- the ratio of AUC cortisol/cortisone.

11.6.10 Analysis of the changes in the AUC for cortisol and cortisone by hour

11.6.10.1 Overall AUC per hour analysis for cortisol, cortisone and cortisol to cortisone ratio

In my analysis, I have also looked at the changes in the AUC per hour. We have excluded the last 2 hours of the 24-hour interval (e.g. the 23rd and 24th hours) because few patients achieved sampling up to this interval. Therefore, the analysis deals with the 1 to 22-hour sampling interval.

In the overall analysis, we found that the AUC for cortisol in the first hour was significantly lower compared to the AUC per hour measured in the interval 4 to 22 hours. This difference was highly significant later, from 6 hours up to 22 hours of sampling ($P<0.0001$). The AUC for cortisol at 2 hours from the start of sampling, were significantly lower compared to the AUC per hour measured in the interval 10-21 hours. This difference was highly significant between 15 and 21 hours of sampling ($P<0.001$) (Figure 93).

11.6.10.2 Analysis of the ratio of AUC for cortisol to cortisone per hour stratified by age, procedure and oxygen saturation

The two-way ANOVA analysis of the change of the ratio of AUC for cortisol to cortisone per hour showed an overall significant effect of the age ($p<0.0001$) and procedure ($p<0.0001$) but there was no significant effect of the ratio of AUC for cortisol to cortisone change per hour nor a significant interaction. In the *posthoc* analysis, there were no significant differences between age groups or procedure groups in the ratios of AUC for cortisol to cortisone per hour. There was no effect of oxygen saturation (cyanotic/acyanotic heart defect) on the ratio of AUC for cortisol to cortisone per hour ($P=0.8094$) (Figure 94).

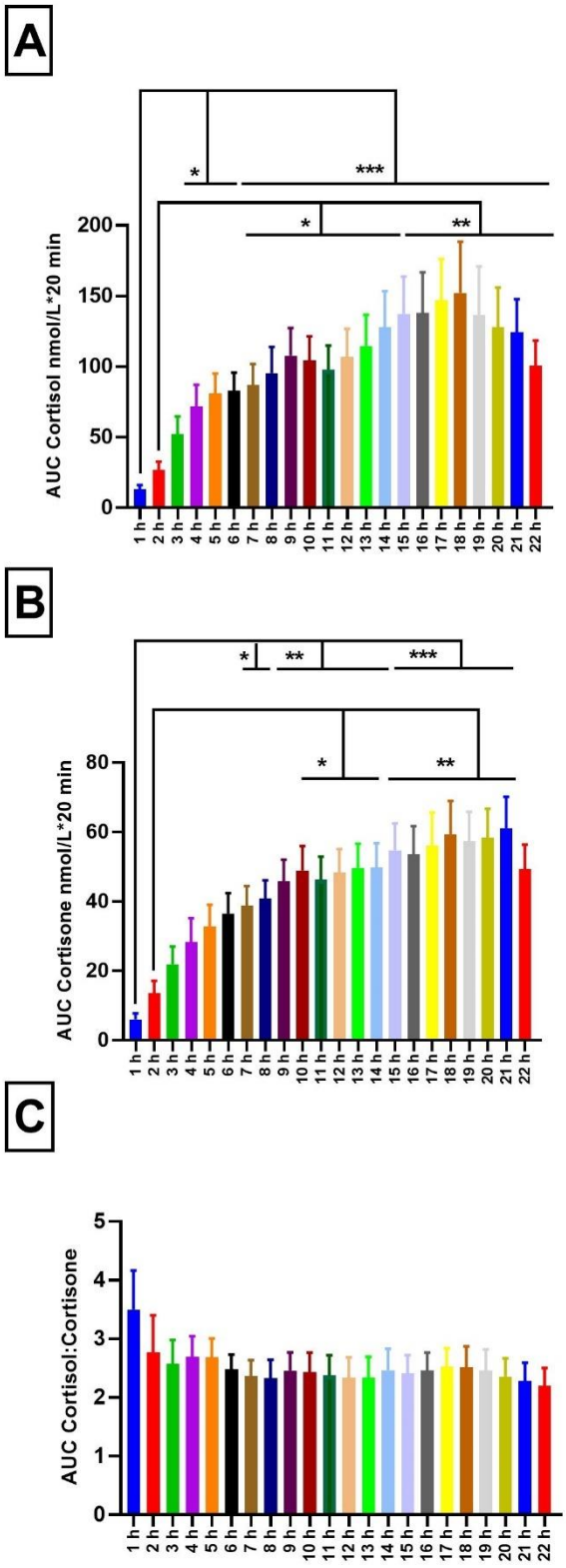

*Figure 93 AUC per hour for cortisol (A), cortisone (B) and the ratio of AUC cortisol to cortisone per hour (C). Data are mean of AUC per hour ±SEM. Data were analysed using a one-way ANOVA test; *P<0.05, **P<0.001 and ***P<0.0001.*

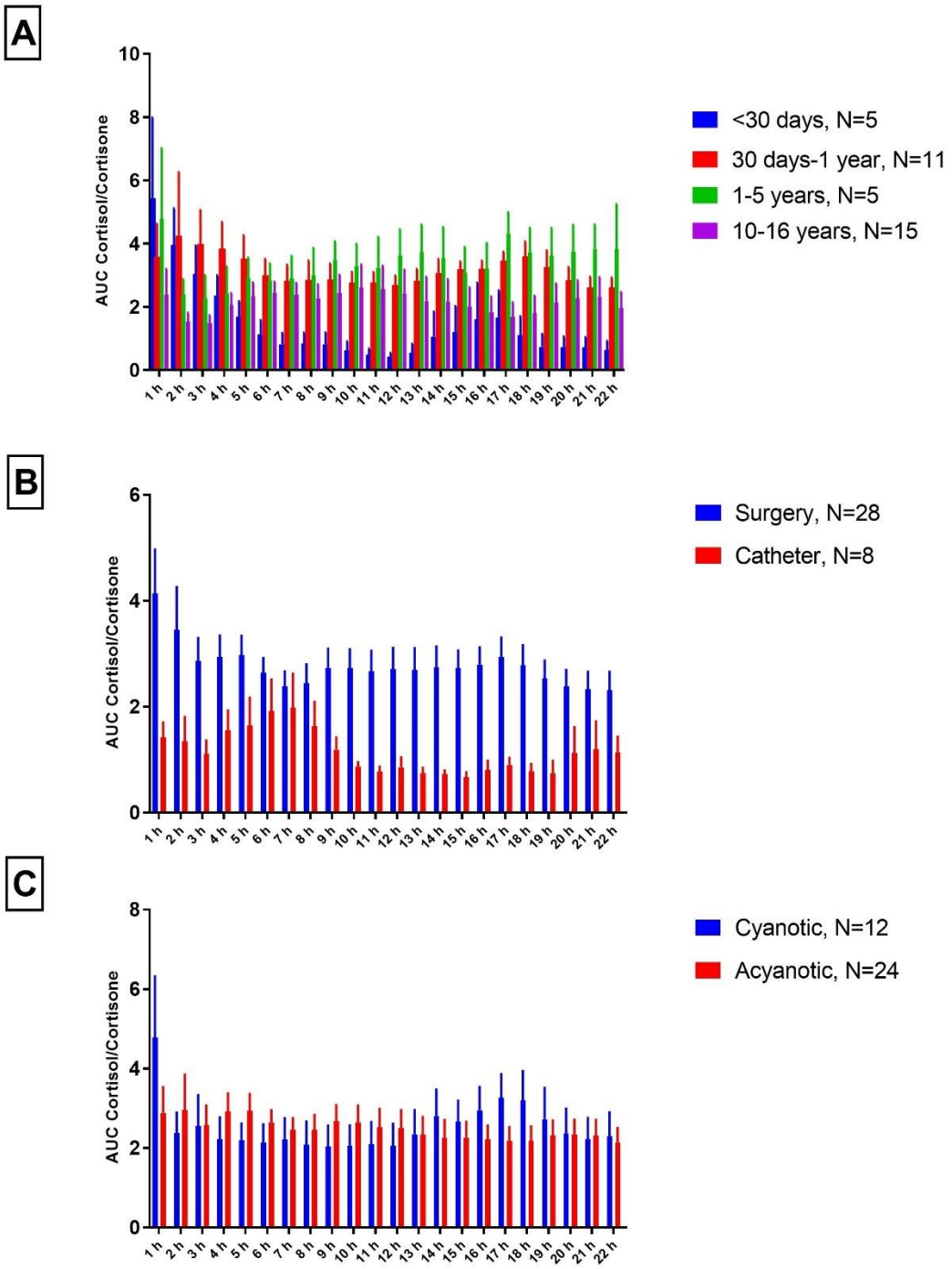

Figure 94 Ratios of AUC per hour by age group (A), procedure type (B) and type of defect. Data are mean of ratios of the AUC for cortisol to cortisone, per hour ± SEM.

11.6.11Changes in serum ACTH and cortisol

The catheter patients had only one baseline serum ACTH and cortisol measurement. The serum ACTH, serum cortisol and ration of ACTH to cortisol are found in Table 14.

Figure 95 A depicts the changes in serum ACTH and cortisol. We could not do a stratified analysis by age, procedure and type of defect (cyanotic/acyanotic) due to missing ACTH and Cortisol data for the various subgroups. Therefore, the data is analysed overall in 20 children that underwent cardiac surgery (1 neonate, 8 infants, 5 children aged 1-5 and 6 teenagers). The ANOVA analysis showed a significant effect of sampling timepoint on the ACTH to cortisol ratio change (P<0.0001). In the *posthoc* analysis, we compared the change in the ACTH to Cortisol ratio to the baseline time point (the before skin incision sampling time point). The analysis revealed a significant increase at the post CPB sampling time point compared to baseline (P=0.0050). Also, the ACTH to cortisol ratio was significantly lower at the 6-24 hours sampling time point compared to the before skin incision timepoint (P=0.0076) (Figure 95 B).

Patient	Cortisol (nmol/L)	Cortisol (µg/L)	ACTH (ng/L)	ACTH/Cortisol (ng/µg)
P22	598	216.7451975	54.3	0.250
P23	375	135.9188112	74.1	0.545
P24	221	80.10148605	36.1	0.450
P25	303	109.8223994	31.4	0.285
P26	203	73.57738311	16.9	0.229
P27	449	162.7401232	58.5	0.359
P28	536	194.2732874	18.2	0.093
P29	344	124.6828561	22.7	0.182

Table 14 Serum ACTH, serum cortisol and ACTH to cortisol ratio for the catheter patients at the baseline sampling time-point

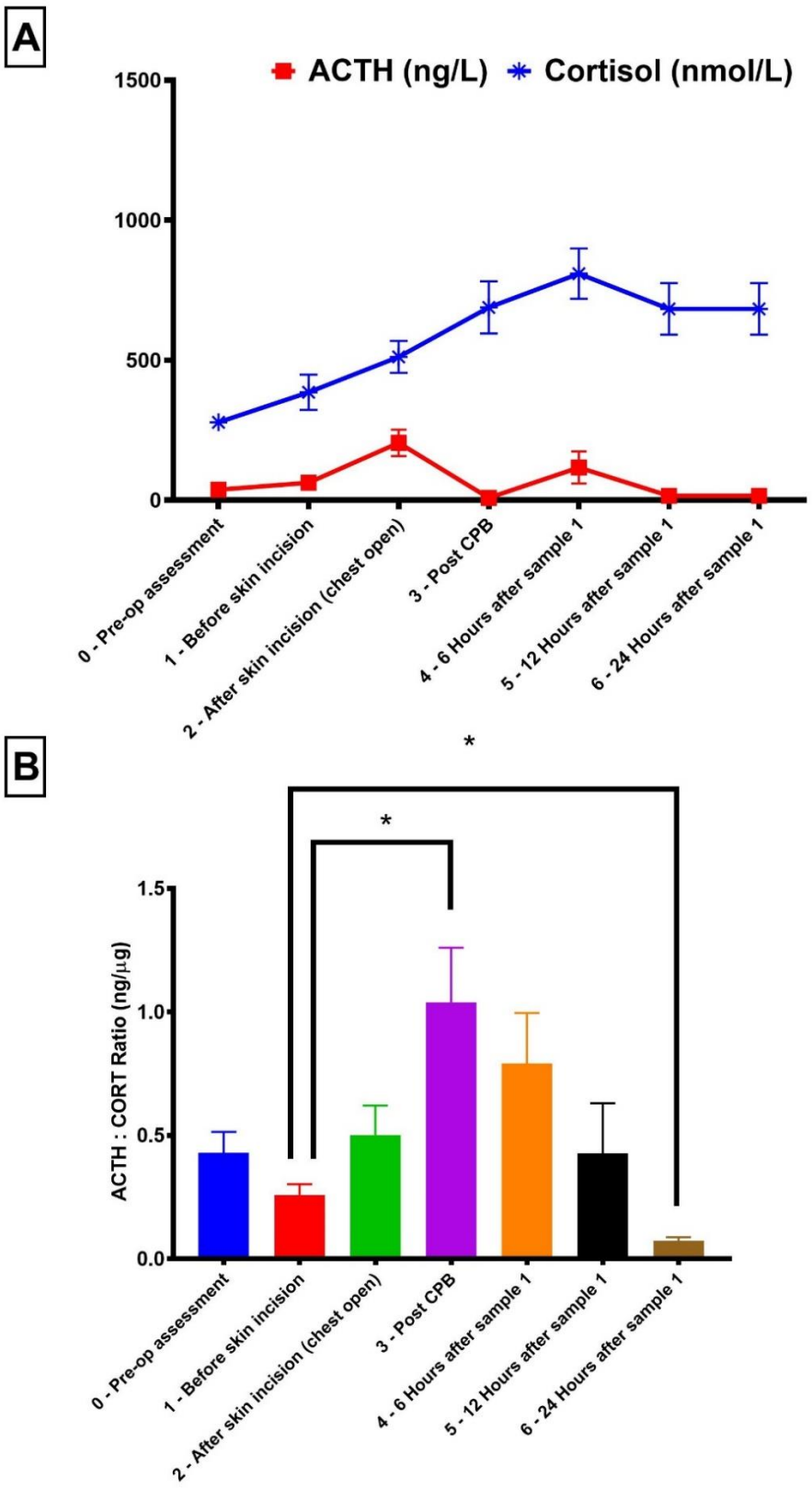

*Figure 95 A - Changes of the serum ACTH in relation to serum cortisol (N=20). Data are mean of serum cortisol (nmol/L) ±SEM and serum ACTH (ng/L); B - Data are mean of ACTH to cortisol ratios (ng/µg) ±SEM; data were analysed using a one-way ANOVA test followed by Kruskal-Wallis post-hoc-test;*P<0.01.*

11.6.12Changes in the CBG perioperatively

11.6.12.1 Overall Analysis

The CBG concentrations were measured at a 1-time point in the catheter patients (Table 15. We had CBG data for 26 surgical patients. In the overall analysis, we found that the CBG concentration significantly decreased at the 6 hours (P<0.0001) and the 24-hour time point (P <0.0001) compared to the preoperative measurements (Figure 96). Similarly, the CBG concentrations at 6 hours (P= 0.0008) and 24 hours (P= 0.0003) were significantly lower. We analysed the CBG perioperative changes by age and type of congenital heart defect in surgical patients.

Patient	CBG µg/mL)
P22	40.4457
P23	54.7962
P24	42.0486
P25	47.5711
P26	53.6462
P27	46.6693
P28	194.771
P29	40.8997

Table 15 CBG at the anaesthesia induction sampling point in the catheter patients

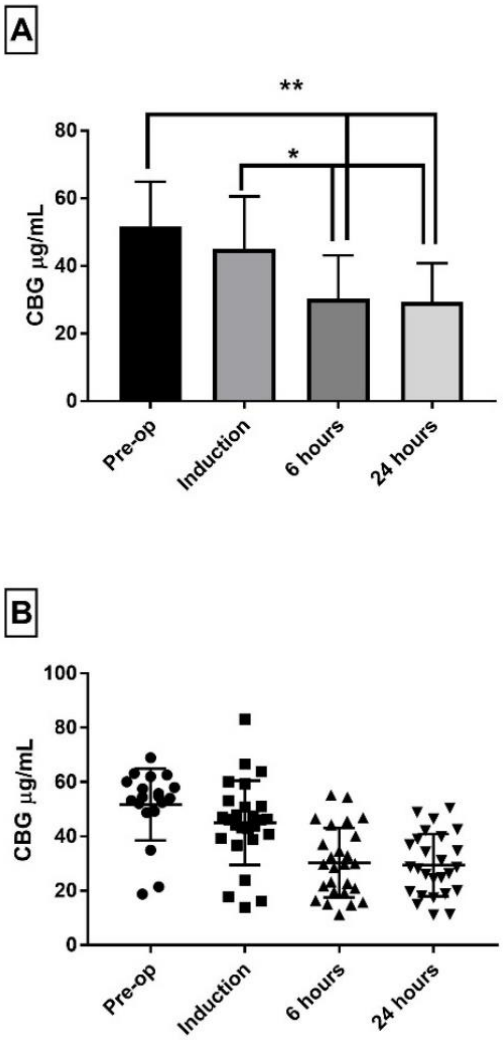

*Figure 96 Overall changes in the CBG perioperatively. A –Data are mean of CBG measurements (ng/mL) ±SEM; data were analysed using the one-way ANOVA test followed by a post hoc analysis using Tuckey's multiple comparisons test; *P<0.001, *p<0.0001, B – the spread of the data at the 4 sampling time points.*

11.6.12.2 CBG analysis by age

In the two-way ANOVA analysis, we found a significant effect of age (P<0.0001) and sampling time (P<0.001) on the CBG concentration. There was a significant interaction between the time of sampling and age (P=0.0054). In the post hoc analysis, we found that the CBG was significantly lower in neonates compared to the rest of the groups at preoperative sampling (P <0.0001) and induction (<0.0001). Furthermore, the CBG in neonates was

significantly lower at 6 hours compared to 1-5 years old children (P=0.0079) and 10-16 years old children (P=0.0036). Similarly, at 24 hours, the CBG concentration in neonates were lower compared to 1-5 years old children (P=0.0031) and 10-16 years old children (P=0.0017). At 6 hours sampling timepoint, the CBG concentration was significantly lower for children aged 30 days-1 years compared to 1-5 years old children (P=0.0134) and 10-16 years old children (P=0.0051). At induction, the 30 days – 1years old children had significantly lower CBG concentration (P=0.0216) compared to children aged 1-5 years while the CBG concentration for 1-5 years old children was significantly higher compared 10-16 years old (0.0177) (Figure 97).

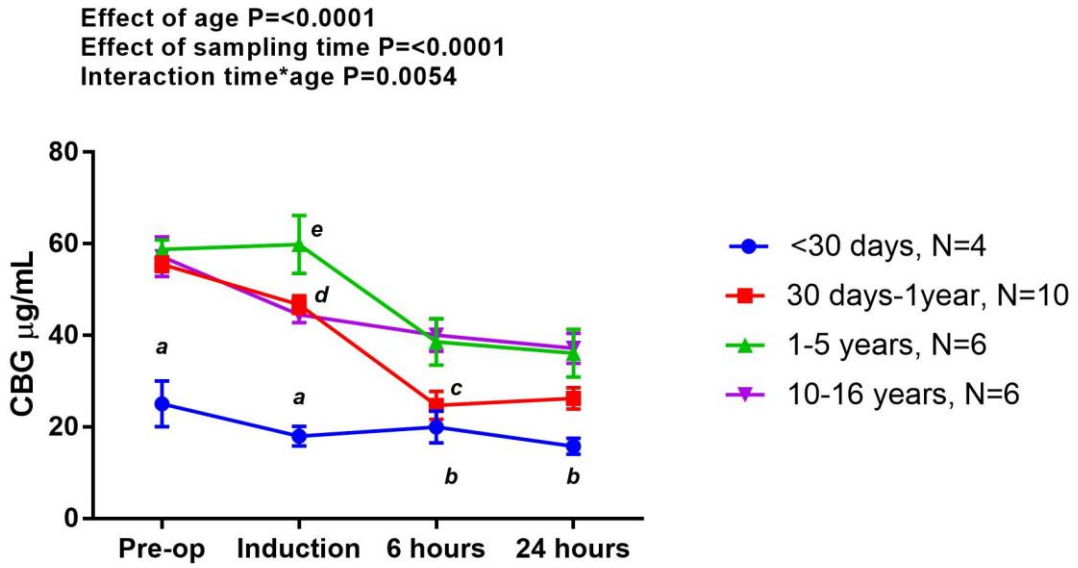

Figure 97 Perioperative changes in the CBG concentration by age group (N=26). Data are mean of CBG measurements (ng/mL) ±SEM; data were analysed using the two-way ANOVA test followed by the Tuckey's multiple comparisons tests; a – P<0.0001, <30 days old children compared to rest of the age groups; b – P<0.01, <30 days old children compared to 1-5 years old and 10-16 years old; c – P<0.05, 30-days old children versus 1-5 years old and 10-16 years old children; d – P<0.05, 30 days-1 years versus 1-5 years old children, e – P<0.05 1-5 years old children versus 10-16 years old children.

11.6.12.3 CBG analysis by saturations

We found a highly statistically significant effect of oxygen saturation (P<0.0001) on the CBG concentrations measured at 6 and 24 hours (P<0.0001). In the post hoc analysis, the CBG concentrations of cyanotic children (oxygen saturation ≤ 89%) were significantly lower at 6 hours (P<0.0151) and 24 hours (P<0.0129) compared to the acyanotic groups (oxygen saturation> 89%) (Figure 98).

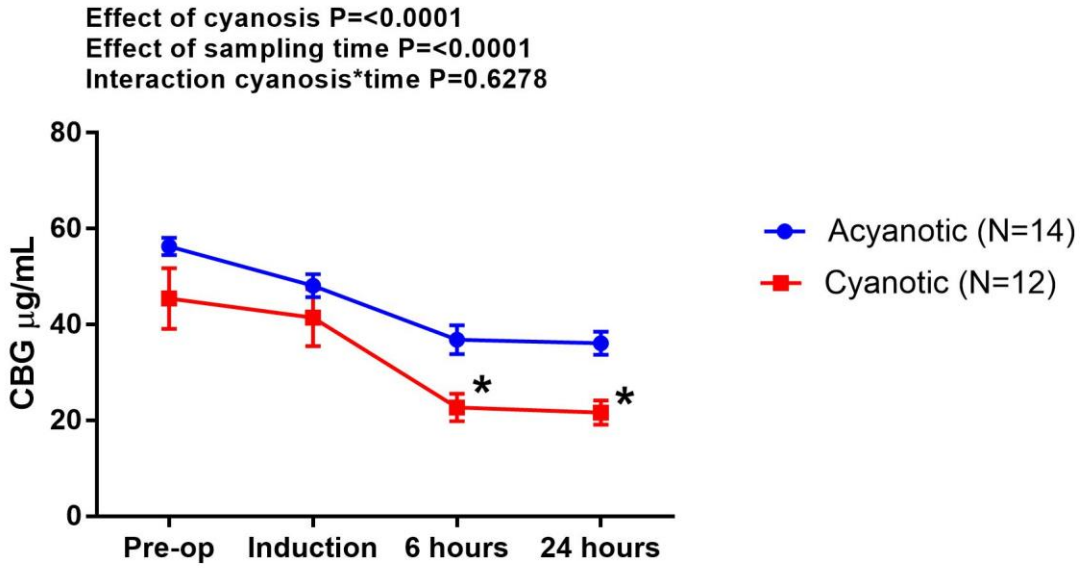

*Figure 98 Pre-operative changes in the CBG concentration by acyanotic versus acyanotic heart defects (N=26). Data are mean of CBG measurements (ng/mL) ±SEM; data were analysed using the two-way ANOVA test followed by the Sidaks's multiple comparisons tests; * P<0.05 acyanotic versus cyanotic.*

11.6.13Preliminary cytokine analysis

This analysis is based on the data available to date (for the surgery group only). This includes cytokine data for one neonate and data for most of the infants, aged 1-5 years and teenagers' group. In the posthoc analysis, we excluded the neonate patient.

11.6.13.1 Cytokine response by age

Analysis of IL-10 showed a significant effect of time of sampling ($P<0.0001$) and a trend towards an effect of age ($P=0.081$) with no significant interaction. In the posthoc analysis, post-CPB, the IL-10 was significantly elevated in the 1-5 years group ($P<0.0001$) compared to the 0, 1, 2- and 24-hours sampling time points. Similarly, IL-10 in the 30 days-1-year groups was significantly higher ($P<0.05$) compared to the 0, 1, 2- and 24-hours sampling time points. Post-CPB, we noted significant age-specific responses. The IL-10 in the 1-5 years group was significantly higher compared to the teenager's group ($P<0.05$) (Figure 99, A). We found no significant changes in the IL-1β response by age (Figure 99, B). There was highly significant age-specific IL-4 response ($P=0.003$) with trends towards a lower production of IL-4 in the 30-days to 1 year compared to older children (1-5 years and teenagers) (Figure 99, C). Analysis of IL-6 showed a significant effect of time ($P<0.001$) but no significant effect on the posthoc analysis (Figure 99, D). Analysis of IL-8 showed a significant of time ($P<0.001$) and age ($P=0.004$) but no significant interaction ($P=0.6121$) and no significant age-specific differences in the posthoc analysis (Figure 99, E). We found a highly significant age-related ($P<0.0001$) TNFα response with a significant of time of sampling ($P=0.0013$). At sampling time points 0 up to 24 hours, we found a significant increase in TNFα, in the 30 days to 1-year group compared to 10-16 years group ($P<0.0005$). Similarly, the TNFα production was significantly higher in the 1-5 years group versus teenagers, between sampling time point 0 to 3 (post-CPB) ($P<0.05$). We have also found a higher TNFα response in 30days – 1-year group compare to the 1-5 years group at the 1 (before skin incision) time point ($P=0.0248$) (Figure 99, F).

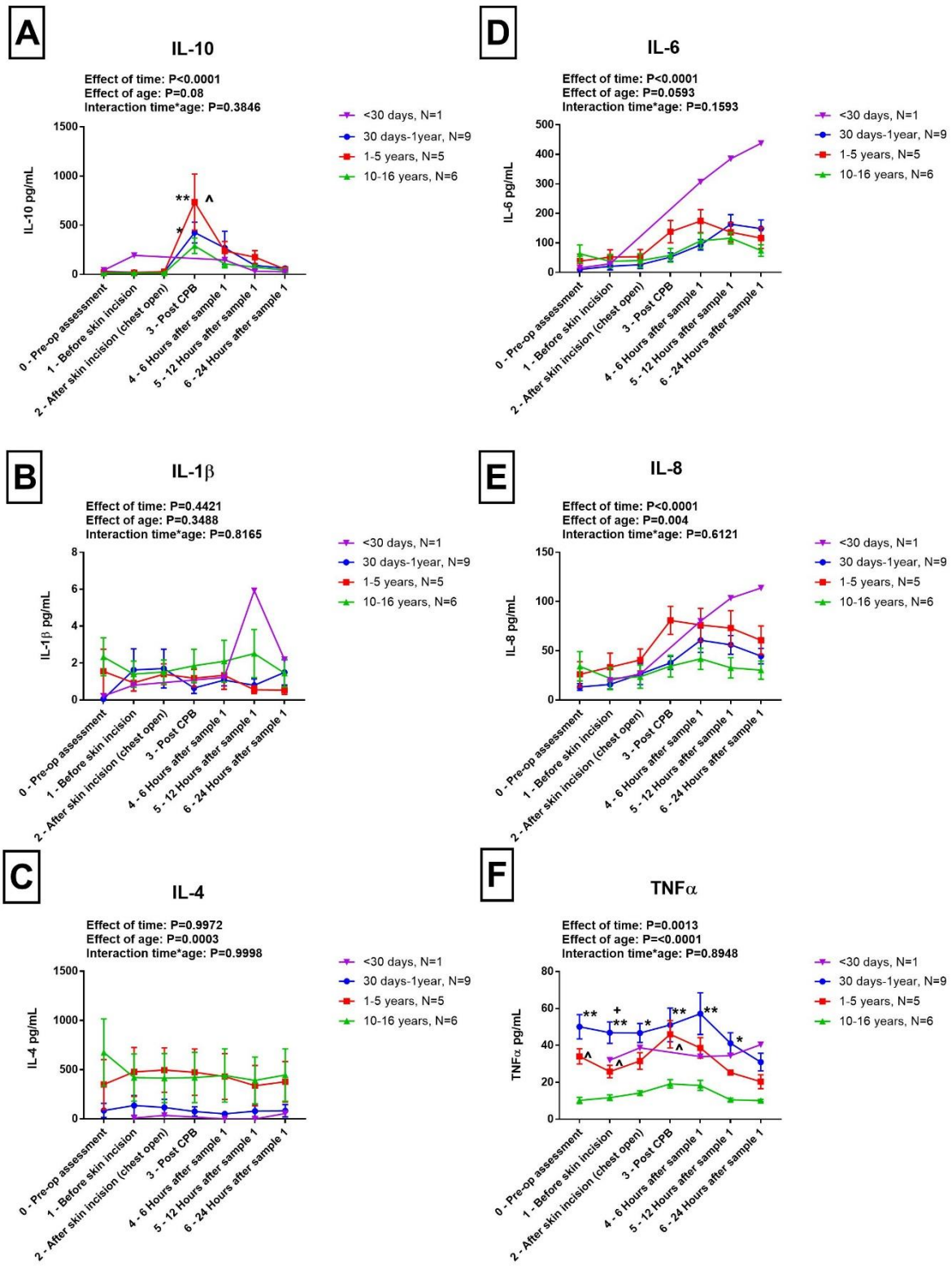

*Figure 99 Changes in cytokine perioperatively depending on age group. Data are mean of cytokine concentrations (pg/mL) ±SEM; Data were analysed using two-way ANOVA test followed by a posthoc Tuckey's multiple comparisons test. A – Changes in IL-10, ** P<0.0001 versus time points 0, 1, 2 and 6 in same age group, *<P0.05; *P<0.05 versus time points 0, 1, 2 and 6 in same age group; ^ P<0.001 age 1-5 versus 30 days- 1year group; Changes in IL-1β, IL-4, IL-6 and IL-8 (B-E); F -Changes in TNFα concentrations; **P<0.0001, 30-days-1 year versus 10-16 years, *P<0.0005, 30-days-1 year versus 10-16 years; ^ P<0.05, 1-5 years versus 10-16 years. $^{+}$P<0.05 30 days-1 year versus 1-5 years.*

11.6.13.2 Cytokine responses by cyanotic versus acyanotic heart disease

We found a highly significant effect of cyanotic heart disease on the production of IL-10 ($P<0.0001$) and a significant effect of time ($P<0.0001$) with highly a significant interaction ($P= 0.0001$). In the posthoc analysis the IL-10 peaked significantly post CPB in the cyanotic group (at sampling time point 3) compared to sampling time point 0, 1, 2, 5 and 6 (P in same age group ($P<0.0001$). This peak at time point 3 was higher for the cyanotic group compared to the acyanotic group ($P<0.0001$) (Figure 100, A). The analysis of IL-1β showed no significant effect of cyanotic heart disease (Figure 100, B). We have also found a significant effect of cyanotic disease on IL-4 production ($P=0.0016$). However, this was not significant in the posthoc analysis for the various sampling time points (Figure 100, C). The IL-6 production was significantly higher for the cyanotic group compared acyanotic patients at the sampling time points 5 ($P= 0.0385$) and 6 ($P=0.0069$). For the cyanotic group, there were significant increases in IL-6 production at time points 4 to 6 compared to the time point 0 and 1 (Figure 100, D). A similar pattern was found for IL-8 that peaked significantly between time points 4 to 5 ($P<0.007$) compared to time points 0, 1 and 2 (Figure 100, E). This increase was significantly higher in the cyanotic group compared to acyanotic group at time point 5 ($P=0.0165$). We found no significant changes in TNFα concentrations.

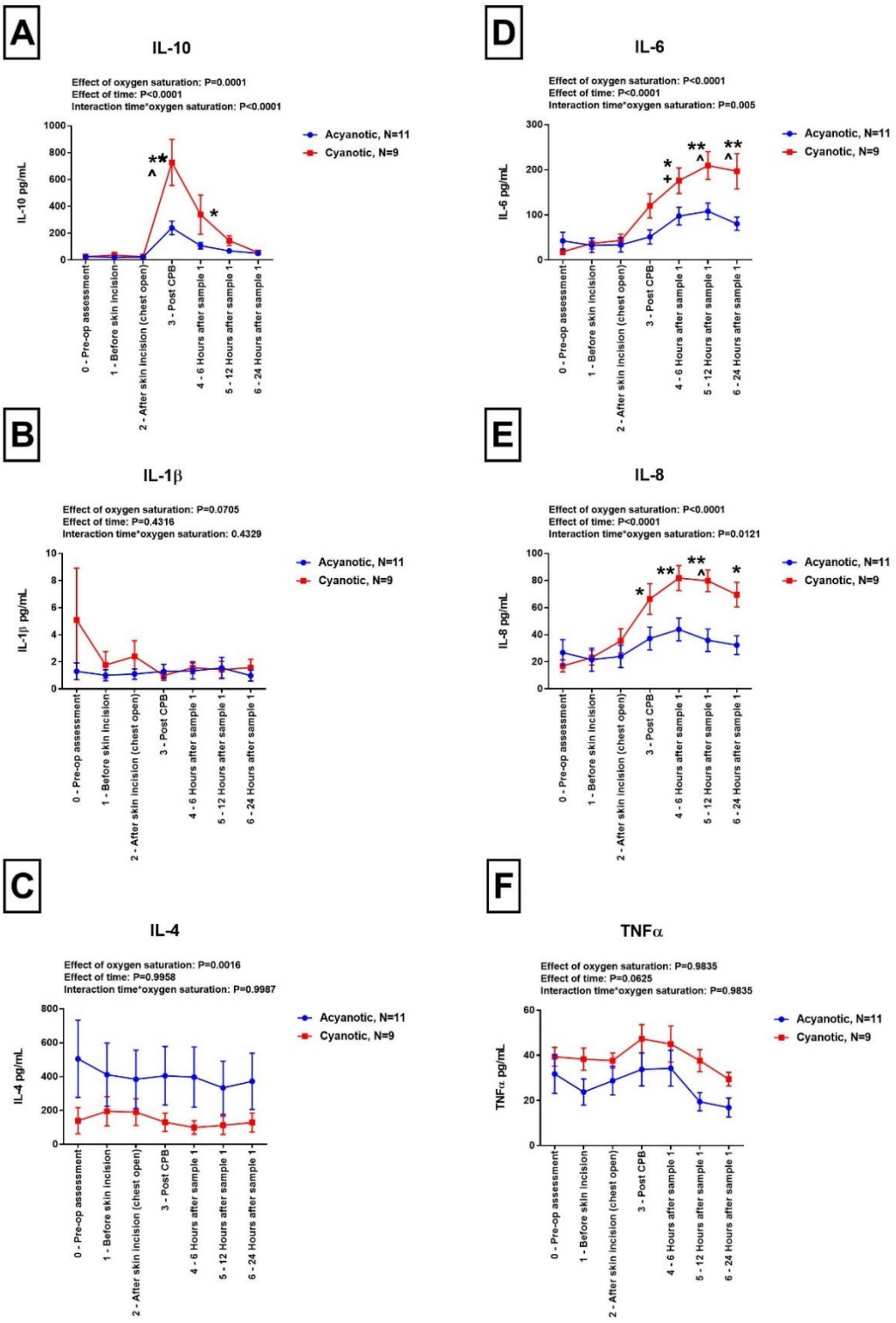

*Figure 100 Changes in cytokine perioperatively depending on acyanotic (O_2 saturation>89%) versus cyanotic heart disease (O_2 saturation ≤80%). Data are mean of cytokine concentrations (pg/mL) ±SEM; Data were analysed using two-way ANOVA test followed by a posthoc Tuckey's multiple comparisons test. A – Changes in IL-10, ** P<0.0001 versus time points 0, 1, 2, 5and 6 time point in same age group, * P<0.001 versus time points 0, 1, 2 and 6; ^ P<0.0001 cyanotic versus acyanotic group. Changes in IL-1β, IL-4 (B and C); D -Changes in IL6 concentrations; **P<0.0001 versus time 0 and 1, *P<0.005 versus time 0 and 1, $^{+}$P=0.005 versus timepoint 2, ^ P<0.05 acyanotic versus acyanotic group. E – Changes in IL-8; ** P≤0.0007 versus time 0, 1 and 2; *P<0.05 versus time 0; ^P<0.05 acyanotic versus cyanotic; F - Changes in TNFα concentrations.*

11.6.14 Discussion

11.6.14.1 Automated tissue microdialysis cortisol measurement in children is feasible

These 36 successful cortisol profiles demonstrate that our automated microdialysis system is a feasible method for the assessment of the HPA axis in children. As outlined before, this method overcomes the limitations of the circulating blood volume in small weight babies and allows measurement of the free active cortisol fraction. Therefore, in the Peacock study, tissue microdialysis was used for the first time to assess the HPA axis response during surgery and it is the first study of this kind in the paediatric population. This allowed us to obtain high-resolution cortisol profiles with up to 70 samples collected every 20 minutes for 24 hours. Even in the patients that were disconnected earlier, we were able to collect more cortisol samples than the previous studies that measured up to a maximum of 10 samples over 48 hours (Table 16).

The main technical disadvantages of the system are (1) the fragility of the microdialysis tubing (2) the microdialysis pump that occasionally malfunctions (3) the long learning curve. All of these issues could be overcome by an improved design and Design Works Ltd (the current manufacturers) is currently working on a new design taking our experience into account. Furthermore, the method can prove feasible to measure multiple steroid hormones (all in the same sample) other hormones such as catecholamines and peptide hormones, metabolomics and drug pharmacokinetics. This is currently work in progress.

Author	Year	Sample size	Study design	Steroids perioperatively	Cortisol time-points	Frequency of cortisol measurement
Kucera et al.(Kucera, Hampl, and Stárka 1986)	1986	24 children	Observational	no data	**6**	1 time-point day before surgery, 4 time-points days of surgery and 1 timepoint 8th day of surgery
Anand et al.(Anand, Hansen, and Hickey 1990)	1990	15 neonates	Observational	no	**7**	pre-op, pre-CPB, during DHCA, end of the operation, 6 hours, 12 hours and 24 hours.
Gajarski et al.(Gajarski et al. 2010)	2010	58 children	Observational	yes	**10**	before surgery, after surgery, 6, 12, 18, 24, 30, 36, 42 and 48 hours
Garcia et al.(Garcia et al. 2010)	2010	21 neonates	Retrospective	yes	**2**	basal and post ACTH test
Mackie et al.(Mackie et al. 2011)	2011	38 neonates	Observational	yes	**3**	preoperative, at 24 hours and 48 hours post-surgery
Wald et al.(Wald et al. 2011a)	2011	52 children	Observational	yes	**2**	pre - and postoperative
Verweij et al.(Verweij et al. 2012)	2012	62 children with low cardiac output	Retrospective	yes	**1**	basal cortisol
Schiller et al.(Schiller et al. 2013)	2013	119 children	Observational	yes	**2**	before and 18 hours after surgery
Banaglore et al.(Bangalore et al. 2014)	2014	33 neonates	Observational	yes	**3**	day 0 (after intensive care unit admission); day 1 (first morning of surgery), day 2 (second morning of surgery)
Crow et al.(Crow et al. 2014b)	2014	32 infants	Observational	yes	**7**	after anaesthesia induction, after CPB, after intensive care unit (ICU) arrival, and 4, 8, 12 and 24 hours after surgery
Teagarden et al.(Teagarden and Mastropietro 2016)	2016	24 patients < 21 years	Retrospective	yes	**1**	pre-hydrocortisone treatment serum cortisol
Maeda et al.(Maeda et al. 2016b)	2016	32 neonates	Retrospective	yes	**3**	baseline and at 30 and 60 minutes after the tetracosactide stimulation

Table 16 Summary of studies to date that have evaluated the HPA axis in paediatric patients

11.6.14.2 The relevance of point cortisol tests in the context of the pulsatile cortisol release – a paradigm shift?

One important finding is that some individual subcutaneous cortisol profiles display a pulsatile pattern. Therefore, the use of single-point cortisol measurements or other tests based on limited time point cortisol measurements (e.g. ACTH stimulation tests) to diagnose the so-called "relative adrenal insufficiency" are difficult to interpret. Therefore, all the body of literature that guides the administration of glucocorticoids based on the current definitions of abnormal adrenal function perioperatively is questionable. These findings also question the relevance of any previous attempts to correlate clinical outcomes with what is believed to be relative adrenal insufficiency.

11.6.14.3 The endogenous cortisol release after surgery

Looking at the cortisol profiles obtained so far, in most cases, the massive release of cortisol that occurs does so several hours after surgery. Firstly, there is an almost hundred-fold increase in the tissue cortisol levels from baseline. This confirms that children do have a significant *endogenous cortisol reserve* that they can mount during and after the stress of surgery. This makes us question again whether adrenal insufficiency after cardiac surgery does exist (D. P. Fudulu, Gibbison, et al. 2018; D. Fudulu, Lightman, et al. 2018). Furthermore, it also makes us question the concept in many centres that all children should be supplemented with exogenous steroids at the cost of multiple short-term and long-term effects (D. Fudulu, Lightman, et al. 2018). We have not noticed any suppressed cortisol profiles in the patients sampled so far. It was also evident that the cortisol release is proportional to the type of procedure (surgery versus catheter). Surgical patients are exposed to a higher insult due to the

surgical incision (midline sternotomy) and exposure to the non-self CPB circuit compared to catheter patients. This is clear on the individual cortisol profiles and the calculation of the AUC by type of procedure (Figure 40-75 and Figure 86).

In the surgical group, we have also observed that the most complex procedures such as Norwood procedures or arterial switch operations were associated with the largest AUC for cortisol. Furthermore, these procedures were performed in neonates, where CPB times were longer compared to older children (Table 9) and therefore the inflammatory stimulus is expected to be greater. When analysed by age group, the teenagers had significantly lower AUC for cortisol than neonates and infants (Figure 85 A). This could be the reflection of the more complicated surgery performed in this group or an age-specific response related to the maturation of the HPA axis.

Secondly, there is a delayed cortisol release in most surgical patients, after the discontinuation of CPB. The cause of this delay in the cortisol surge of several hours is unclear. It is, however, similar to the adult cardiac surgical plasma cortisol data by Gibbison *et al*. (Ben Gibbison et al. 2015). This delay is longer than would be expected simply due to the need for cortisol to be manufactured *de novo*. This could reflect the fact that activation of the HPA axis, in this situation, is secondary to cytokine release (see cytokine discussion). It is unclear, however, why other patients have a cortisol peak that coincides with surgery or begins just immediately after surgery (Section 11.6.13: P1, 3, 4, 6, 7, 14, 19, 23, 26, 27, 28). A potential explanation could be priming of the adrenal response due to the pre-existing pathology.

11.6.14.4 Overview of the cortisol to cortisone relation during stress

This equilibrium between cortisol and biologically inactive cortisone is maintained by the 11ß-HSD enzyme system (Figure 101). The 11ß-HSD enzyme type 2 (dehydrogenase enzyme) converts the active cortisol into cortisone in the kidney, colon, placenta, fetus, salivary glands) while the type 1 11ß-HSD (a reductase) regenerates cortisone back to active cortisol in tissues such liver, adipose tissue, lung. During normal physiological conditions, the system favours cortisol production over cortisone, maintaining a ratio of about 5:1 (Vogeser et al. 2003; 1999).

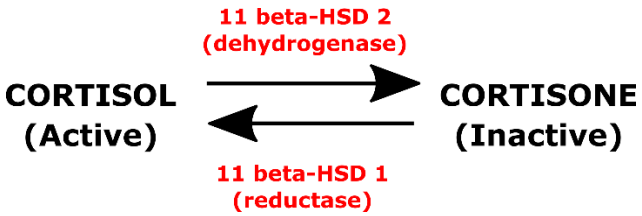

Figure 101 11ß-HSD isoenzyme system

At the conception of the study, we did not plan to measure ***cortisone*** in addition to cortisol in the microdialysate. Because the very sensitive cortisol analysis done in collaboration with Groningen permitted us to measure this too, on relatively small volume per sample, we decided to add cortisone levels as a measure of the activity of the 11β-Hydroxysteroid dehydrogenase system (11ß-HSD). Interestingly we noticed a dynamic change in the ratio of cortisol to cortisone over time and in p particular an ***increase in the cortisol to cortisone ratio*** following major secretory episodes of cortisol.

This suggests that the increased cortisol to cortisone tissue ratio could either be due to saturation of the 11ß-HSD type 2 system by the massive release of cortisol or it could be due to altered regulation of one or both the 11ß-HSD enzymes. Thus, either activation of 11ß-HSD

type 1 (mainly in the liver) or inactivation of the 11ß-HSD type 2 (mainly in the kidneys) could alter the cortisol to cortisone ratio in this way.

In our data, catheter procedures were associated with lower total AUC cortisol to cortisone ratios compared to surgery. This suggests that in the context of the massive release of cortisol during surgery, the 11ß-HSD is overwhelmed and does not convert as efficiently cortisol to cortisone (Figure 90, C).

Several studies have demonstrated a similar increase in the cortisol to cortisone ratios in the days after surgery (Vogeser et al. 2003; Heckmann et al. 2014). An earlier study by Vogeser *et al.* (Vogeser et al. 1999) that measured the urinary cortisol and cortisone metabolites pointed towards a reduced cortisol inactivation (e.g. inhibition of the dehydrogenase enzyme isoform). Reduced reduction of the 4,5-double bond of the A-ring of cortisol and cortisone (predominantly by the liver) was found to play a role in the critically ill patient(Heckmann et al. 2014). However, it is unlikely that this plays a role in the acute response. If we hypothesise that during the acute phase of the stress response, there is inhibition of the 11ß-HSD type 2 in the kidney, this mechanism can have huge implications in the perioperative retention of fluid (Figure 102). If this is the case, this means that some children could develop perioperatively a so-called ***syndrome of apparent mineralocorticoid excess*** as has been described by Weber in 1974. This causes a severe form of low-renin, low-aldosterone hypertension as a result of a mutation in the HSD11B2 gene, causing 11ß-HSD type 2 deficiency(Ferrari and Krozowski 2000). The pathophysiology of this syndrome has been well described (Ferrari and Krozowski 2000).

Cortisol circulates at a 100- to 1000-fold higher level than aldosterone. Both cortisol and aldosterone compete for the mineralocorticoid receptor. The role of the 11ß-HSD type II in the kidney is to selectively convert cortisol to inactive cortisone so that aldosterone can freely exert its physiological effects. If the enzyme activity is inhibited or even if its local

activity is saturated at such high levels of cortisol, then cortisol will exert a mineralocorticoid effect resulting in fluid retention (retention of Na^+ and excretion of K^+) in the absence of raised renin. The syndrome could be reversed by treatment with MR antagonist – *spironolactone*. This has enormous implications for clinical practice because it was always thought that the fluid retention, we encounter in the postoperative patient is related to an activation of the renin-angiotensin system. It is well known that fluid retention after paediatric heart surgery with the use of CPB is associated with worse outcomes (Hassinger, Wald, and Goodman 2014; Jonas et al. 2003).

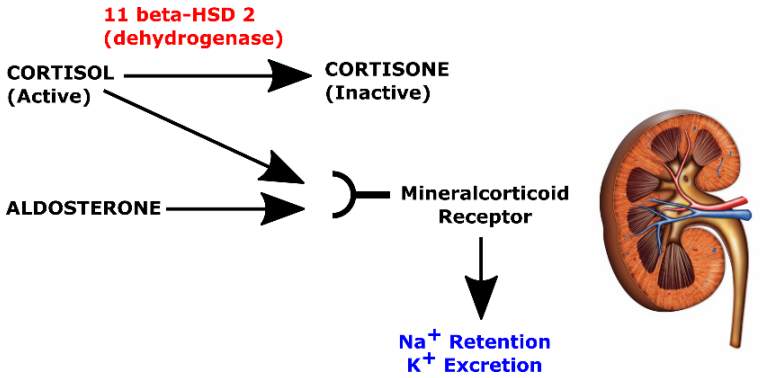

Figure 102 Effect of the 11ß-HSD type 2 in the kidney

11.6.14.5 Patterns of cortisol and cortisone release depending on the age of the patient, type of surgery and type of congenital heart defect

The patterns of cortisol and cortisone release were different in neonates (Figure 89) compared to older children in that neonates had higher ratios of cortisol to cortisone. This finding suggests a higher ability of neonates to convert cortisol to cortisone. The mechanism can be explained, as outlined in the previous section, either by increased activity of the (1) 11ß-HSD-2 enzymes, or (2) reduced activity of 11ß-HSD-1 or both. Several mechanisms can be responsible for these changes in the new-born (Figure 103). Not only the placenta is rich is in 11ß-HSD 2 protecting the fetal development by inactivation of the maternal cortisol to

cortisone (Cottrell et al. 2014) but fetal tissues are also rich in 11ß-HSD 2 enzyme (Chapman, Holmes, and Seckl 2013). Another contributing mechanism is that the activity of the 11ß-HSD 1 is switched on later in the life of the baby and after birth there is not much activity of the enzyme (Speirs, Seckl, and Brown 2004) This is contained mainly in the liver and due to its high organ mass could be mainly responsible for the increased conversion to cortisol later in life. Finally, we did not have enough data show that a distinct cytokine response in neonates compared to older children (please see Cytokine discussion section). However, several studies have shown that the activity of type 1 11ß-HSD is enhanced by inflammatory cytokines such as IL-1 and TNF-α (Escher et al. 1997; Cai et al. 2001).

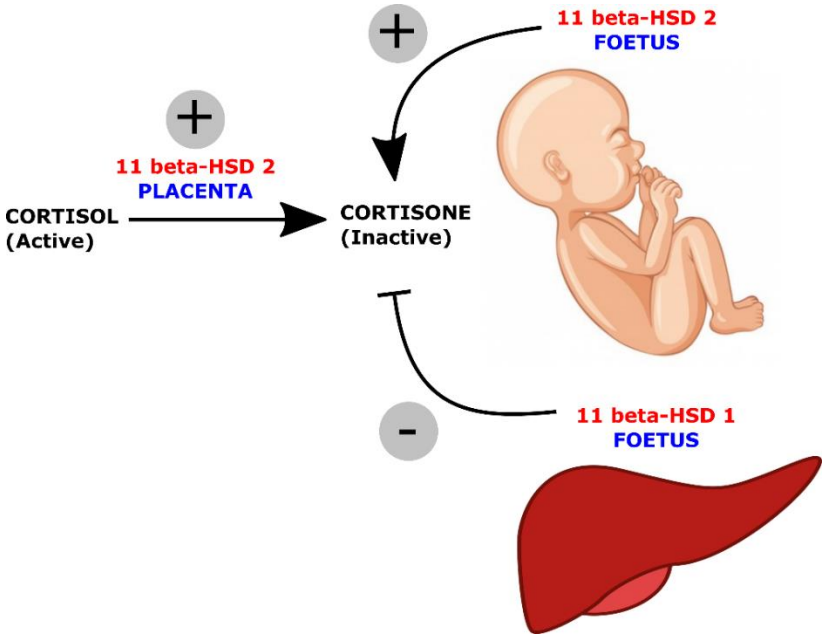

Figure 103 11ß-HSD 1 and 2 in the fetus

11.6.14.6 ACTH and serum cortisol data

Another aspect that is worth discussing is the correlation of the ACTH and cortisol plasma with the tissue cortisol levels. This correlation is quite challenging to ascertain since

we have measured ACTH and serum cortisol at only a few reference time-points. However, some profiles display no apparent correlation between plasma and tissue cortisol levels (Section 11.6.13: P9, P10, P16, P19, P23, P24, P26). If we look at the plasma ACTH and serum and tissue cortisol, the data is similar to Gibbison et al. (Ben Gibbison et al. 2015) studies where after the initial ACTH increase, the cortisol remains elevated while the ACTH levels are reduced. This possibly suggests an increased sensitivity of the adrenal gland to ACTH in children as seen in adults. This is reflected in the analysis of the ACTH to cortisol ratio, where we found the ratio to be significantly increased at CPB compared to pre-surgery and significantly decreased to pre-surgery levels up to 24 hours.

11.6.14.7 Plasma CBG concentrations

In the overall analysis, the CBG levels dropped significantly postoperatively (at 6 and 24 hours) compared to levels measured at induction (Figure 96). This finding is similar to previous studies in adults (Ben Gibbison et al. 2015; Roth-Isigkeit, Dibbelt, and Schmucker 2000) and children (Mcmaster et al. 2010) and most likely the cause is haemodilution (Roth-Isigkeit, Dibbelt, and Schmucker 2000). However, in the context of major surgery, this could also be due to variation in temperature, neutrophil count or pH (Cameron et al. 2010; D.E. Henley and Lightman 2011; Hammond et al. 1990). Due to the finite ability of CBG to bind cortisol in plasma (Ballard 1979) and in the context of a pulsatile secretion, this results in vast swings of free, available, cortisol that can act on the GR receptor. However, one interesting finding is the influence of age on CBG concentrations of patients having surgery. Neonates displayed significantly lower levels of CBG compared to older, at all-time points. We noted further age-related differences, with a trend towards lower CBG levels the younger the age group. A potential explanation is that younger children are sicker (turned off CBG) and

received more intravenous fluid or this could be an age-specific response. Moreover, in the analysis by cyanotic versus acyanotic heart disease, the CBG levels were significantly lower in the cyanotic patients at 6- and 24-hour time points compared to the acyanotic patients (Figure 98). In the study by Wald et al. (Wald et al. 2011b), the authors noted the significant variation of CBG levels across a cohort of 51 patients with ages ranging from 0.4 to 6.5 years and this could have been the effect of the age and the underlying congenital heart defect.

11.6.14.8 Characterisation of the inflammatory response

The paediatric inflammatory response to CPB has been studied more than the adrenal response. However, as stated before, its relation to clinical outcomes is unclear (D. Fudulu, Lightman, et al. 2018; D. P. Fudulu, Gibbison, et al. 2018). Furthermore, the relation of the cytokine response to HPA axis function during and after paediatric heart surgery is not well understood. This will be a direction of the analysis once recruitment has finalised. On the preliminary analysis of the cytokine by age and underlying congenital disability, we noted significant changes in IL-10, IL-6, IL-8 and TNFα levels and this in keeping with previous studies (Madhok et al. 2006; Alcaraz et al. 2002; 2005). We noted a significant increase of the IL-10 immediately post CPB in age groups 30 days-1-year-old children and 1-5 years old children. This increase was the most significant in the 1-5 years old group. In this preliminary analysis, we excluded the neonate for whom we had cytokine data; hence, it is difficult to draw any comparisons with other age groups.

A limitation of the study is not measuring cytokines in neonates at the post-CPB timepoint, where we noted most changes IL-10 to occur. However, Alcaraz et al. (Alcaraz et al. 2002) showed that this immediate increase is more profound in neonates that secrete more IL-10 compared to older children. Since IL-10 is a major pro-inflammatory cytokine, its role

is to temper the inflammatory response post-by-pass. Interestingly, in the analysis by oxygen saturation, the IL-10 was significantly more increased post-bypass in the cyanotic children compared to the acyanotic children. Hövels-Gürich et al. (Hövels-Gürich et al. 2002) found that the levels Il-10 were lower pre-operatively in infants with hypoxemia owing to intracardiac right-to-left shunt compared to infants with heart failure because of an intracardiac left-to-right shunt. We found no difference at baseline in our study. The maximal rise of IL-10 occurred post-CPB immediately in our study, and this was similar to the above study where the maximal rise was at the protamine administration (i.e. termination of CPB) time-point. All in all, these findings suggest that IL-10 is rapidly secreted post-CPB to attenuate the inflammatory response. This is more pronounced in the most vulnerable groups: younger children and cyanotic patients. We found no significant changes in IL-1β, which were similar to previous studies (Madhok et al. 2006). There is not much literature investigating the effect on the pro-inflammatory cytokine IL-4. However, we noted an effect of age of the child with a trend towards higher levels in older children and lower levels in the cyanotic patients compared to acyanotic. We also noted that the pro-inflammatory cytokine levels for IL-6 and IL-8 started to rise post CPB, with a peak for IL-6 at 12 hours and at 6 hours for IL-8. This suggests that the pro-inflammatory cytokine IL-10 rises immediately after by-pass and then returns to baseline while the pro-inflammatory cytokine response is more delayed and kicks in several hours after surgery. We found no effect on age on IL-6 and IL-8 however, both cytokines were significantly more elevated in the cyanotic patients. Alcaraz et al. found the same peak of IL-6 and IL-8 to occur immediately post-CPB (Alcaraz et al. 2005). In addition, neonates had a distinctive response in that they secreted higher levels of IL-8 compared to older children. In the same study, IL-8 levels were significantly correlated with postoperative morbidity (pulmonary dysfunction, days on inotropic support and days of PICU stay). Moderate increases in TNFα within 24 hours from surgery have been reported by previous studies (Khabar et al.

1997; Alcaraz et al. 2005) with a higher response in the neonates (Alcaraz et al. 2005). Again, we included in the preliminary analysis only one neonate; however, we noted a strong effect of age on the pattern of TNFα release in that younger children (infants and 1-5 years old) produced higher levels than teenagers.

Finally, we have to acknowledge several limitations in the evaluation of the cytokine response. Firstly, it will always be challenging to discern the magnitude of the effect of surgery on the individual cytokine release from an age-specific response. Secondly, some of the children had modified ultrafiltration at the end of CPB; hence, this is known to affect the levels of specific cytokines (mainly TNFα) (Brancaccio et al. 2005).

Interleukin 1, TNFα and IL-6 are known to activate the HPA axis (Turnbull and Rivier 1995). On our preliminary data, IL-1β had no significant changes, and the increases in TNFα were modest and non-significant compared to pre-op levels. However, we noted significant increases in IL-6 at post CPB at 6, 12 and 24 hours from the start of the operation. As discussed in the previous chapters, IL-6 is known to be a potent activator of the HPA axis (Bethin, Vogt, and Muglia 2000) This coincides with the cortisol peaks that we noted to occur within several hours after the end of surgery. This fits with previous hypotheses that suggest a delayed HPA axis activation mediated by cytokines (B Gibbison et al. 2015), but we were unable to specifically test this hypothesis.

11.6.15 Considerations on microdialysis cortisol profiles of glucocorticoid recipients.

Two patients received glucocorticoids during microdialysis sampling and were withdrawn (Figure 80, Figure 81, Figure 82). One patient was a 4 years old boy that had bidirectional Glenn shunt and double aortic arch division (P 37) and received dexamethasone (0.25mg/kg, IV) to prevent airway oedema post-extubation because of pre-operative bilateral vocal cord palsy (Figure 80). The other child was a 3-week-old boy (P38) that underwent arterial switch procedure and atrial septal defect closure for transposition of great arteries. He had hydrocortisone (2 mg/kg, IV) for low cardiac output syndrome and increased vasopressor requirement. Pre glucocorticoid administration, there was no suppression of the tissue cortisol response to surgery in both patients. The dexamethasone recipient displayed suppression of the ACTH level post dexamethasone administration with a remarkable lack or delay of suppression of cortisol, suggesting autonomous activation of cortisol release. (Figure 80). The hydrocortisone recipient had a steep, supraphysiological increase of the tissue cortisol levels (peak level of 1839 from a basal level of 1.08) and suppressed ACTH levels (Figure 81 and Figure 82) . This patient is currently alive; however, he had a ventilation time of 6 days and a long admission of approximately 1 month. The postoperative recovery was complicated by acute kidney injury for which he required peritoneal dialysis in PICU. The time of administration (on the prescription) correlated very well with the time we recorded the tissue cortisol increase from the start of the device. This demonstrates again good correlation of intravenous cortisol levels with the tissue cortisol levels that increased shortly after intravenous administration.

11.6.16 Conclusions

The preliminary data from the Peacock Study firstly demonstrates that automated tissue microdialysis sampling method was feasible to measure cortisol in children of various ages. We have shown that tissue cortisol levels display a pulsatile pattern–throughout the perioperative period. This confirms the inappropriateness of single point cortisol tests. Neonates were a distinct group by the pattern for cortisol to cortisone release. The data suggests that they have a higher ability to convert cortisol to cortisone. Probably, this reflects a higher activity of the 11- HSD type 2 enzyme and/or immaturity of the 11β-HSD type 1. As expected, we found patients undergoing heart surgery had higher levels of cortisol compared to catheter patients with lower cortisol to cortisone ratios suggesting substrate overload of 11- HSD type 2 enzyme. There was no significant effect of cyanosis on cortisol secretion.

Similar to adult studies, the serum ACTH and cortisol levels suggest an increased adrenal sensitivity to ACTH. Overall, the CBG levels were reduced perioperatively in line with previous studies in adult and children. However, we found variations in CBG concentration by age and type of defect (cyanotic/acyanotic). Younger children and cyanotic patients had lower levels of CBG, suggesting a higher availability of free cortisol in these patient groups. We found age-related and cyanosis related changes in cytokine secretion patterns during and after surgery.

11.6.17 Suggestions for future work

The short-term objectives are to finalise recruitment in the Peacock study. To date, there are nine more surgical patients (10-16 years group) 18 more catheter patients (mostly 30 years-1 year and 1-5 years) to be recruited.

A more in-depth analysis of all the data is needed. A possible approach is to use machine learning techniques to deal with this amount of data. If the time-series data is aligned in a meaningful way, this can allow identifying repeated features and potential anomalies. These can be further classified using clustering and decision tree analysis. Ideally, the model would integrate the dynamic microdialysis data with all the serum measurements (serum ACTH, cortisol, cytokines) and the clinical outcome data to predict how children of various age groups would respond to surgery.

A more long-term objective would be to understand the 11β-HSD enzyme interplay responsible for the cortisol to cortisone ratio increase, and this would ideally involve measurement of type 1 and type 2 enzyme 11β -HSD enzyme activity in children, particularly in neonates or in an animal translation model (pig). This could be investigated by measuring plasma cortisol and cortisone concentration, urinary cortisol, cortisone and specific metabolites and couple this with measurement of the changes of serum Na, K, aldosterone and renin activity.

12 OVERALL DISSCUSSION, CONCLUSIONS AND LIMITATIONS

12.1 Overall discussion

To understand the HPA axis response of children of various ages, I have used the stress model of paediatric heart surgery. The results of my thesis highlight how complex the paediatric HPA axis regulation is during acute stress and how little we know about this subject. Children display an endogenous cortisol response proportional to the stress insult. Paediatric cardiac surgery with the use of CPB resulted in a more profound cortisol release compared to the lower invasive catheter procedures. The cortisol patterns were pulsatile, and this suggests that single point cortisol tests are not accurate in defining perioperative adrenal dysfunction. In the context of acute stress, the adrenal regulation is not only modulated by pituitary inputs. As shown in my in vitro work, the immune cells exert a local control and can change the activity of the steroidogenic pathway. These local mechanisms are likely complementing the HPA axis activation during stress. Furthermore, this local interaction can be directly modulated by the effect of glucocorticoids.

My data shows significant postoperative changes in CBG concentration. This can further have profound changes in the availability of free plasma cortisol during the stress response to surgery.

The changes in the cortisol to cortisone ratios we have noted are likely the effect of a change in the 11-β-HSD enzyme system activity. This finding highlights that the availability of cortisol during stress is also regulated to a great extent at the tissue level. This regulation appears to be age and procedure-related. Neonates are a distinct physiological group because of a higher conversion of cortisol to cortisone. Furthermore, the cytokine response patterns are age-specific and influenced by the type of congenital defect (chronic hypoxia). Therefore, if

anti-inflammatory steroids are indicated in children undergoing heart surgery, a “one-size-fits-all” approach is likely not to be useful.

12.2 Overall conclusions

I demonstrate that our basic understanding of the physiology of the hypothalamic-pituitary-adrenal axis of children undergoing paediatric heart surgery is very limited. Therefore, by using an automated tissue microdialysis system in children, I show that cortisol is released in a pulsatile manner during and after surgery. Furthermore, I found a change in tissue cortisol metabolism, suggesting an involvement of the 11 β-hydroxysteroid dehydrogenases. The cortisol release was also accompanied by an early systemic release of anti-inflammatory cytokines and a more delayed release of proinflammatory cytokines. By using a novel co-culture model, I also demonstrate how a local adrenal crosstalk between the immune and adrenal cells can modulate the expression of adrenal cytokines and the activity of the steroidogenic pathway during acute stress.

12.3 Limitations

My PhD thesis has several limitations I would like to acknowledge in this section.

With regards to the in vitro work, the cell lines used originated from adult rats and human adults, respectively. Hence, we could not study the changes in immune-adrenal interactions according to the age of the cells. Another limitation of using this in vitro model is that we do not understand to what extent these observations apply to the in-vivo immune-adrenal interactions that could prove more complex.

Concerning the Peacock Study, the main technical disadvantages of the system are (1) the fragility of the microdialysis tubing (2) the microdialysis pump that occasionally malfunctions and (3) the long learning curve. All of these can be overcome by an improved design and DesignWorks Ltd (the current manufacturers) are currently working on a new design taking all our experience into account.

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